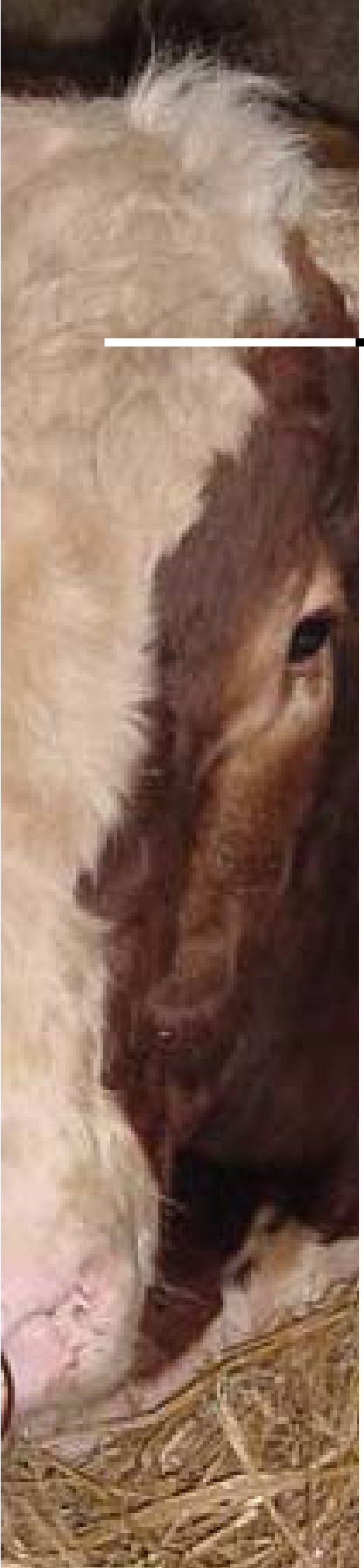

Bovine semen preservation under epididymal conditions and assessment of sperm quality by means of a sperm-oviduct binding assay

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TABLE OF CONTENTS

List of abbreviations

List of abbreviations

AI	artificial insemination
ATP	adenosine triphosphate
BSA	bovine serum albumin
CASA	computer assisted sperm analysis
CEP	cauda epididymal plasma
CI	confidence interval
CTC	chlortetracycline
COC	cumulus oocyte complex
DHT	dihydrotestosterone
2D-SDS-PAGE	two-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis
DNA	deoxyribonucleic acid
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
Hepes	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HF	Holstein Friesian
hpi	hours post insemination
HZA	hemizona binding assay
IVF	in vitro fertilization
IVM	in vitro maturation
JC-1	5,5',6,6'-tetrachloro-1,1'13,3'-tetraethylbenzimidazolyl carbocyanine iodide
LDL	low-density lipoproteins
MW	molecular weight
NRR	non-return rate
PBS	phosphate buffered saline
PCM	principal cell medium
pH_i	intracellular pH
PI	propidium iodide
PI	isoelectric point
PS	phosphatidylserine
PSA	Pisum sativum agglutinin
PNA	Peanut agglutinin
PVP	polyvinyl-pyrrolidone
r	correlation coefficient
ROS	reactive oxygen species
RT	room temperature
SD	standard deviation
SEM	standard error of the mean
TALP	tyrode solution supplemented with albumin, lactate, pyruvate
TCM	tissue culture medium
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling
UV	ultraviolet
ZBA	zona binding assay
ZP	zona pellucida

1.

INTRODUCTION

1.1 History of sperm preservation

1.2 Aims of the study

1.1 History of sperm preservation

The spread of genital diseases by natural service is a constant threat in bovine. Moreover, keeping herd bulls is expensive and represents a potential danger for the herd manager. From this point of view, the artificial insemination (AI) industry has developed at the cost of natural service over the past 50 years. Artificial insemination is now used in almost every country in the world (Thibier and Guerin, 2000). One of the main factors contributing to its success is the rapid and widespread diffusion of improved genotypes and the exchange of genotypes without transmitting diseases, so that AI can be performed without risks (Leboeuf et al., 2000). This is mainly due to the high standard of health surveillance at the AI centers. Artificial insemination has now a major impact on cattle breeding, through its use of bulls of high genetic merit and by selective rearing of calves of high breeding merit (Vishwanath and Shannon, 2000). While most semen is processed for use in AI, semen can also be stored for the preservation of genetic material of endangered species, as part of an active conservation program (Thibier and Guerin, 2000).

The demand for semen from bulls of high genetic merit has been the main motivation for developing and refining storage technologies (Vishwanath and Shannon, 2000). In most developed countries, AI was introduced on a small scale during the 1940s and 1950s and carried out with fresh semen or semen stored at room temperature (RT). During the 1960s deep-frozen semen started to be processed while donor agencies encouraged the introduction of highly specialized and expensive AI establishments. A more recent survey has shown that the total number of doses of semen produced exceeded 200 million, with more than 95% processed as a frozen product (Chupin and Thibier, 1995). Current tendencies in some countries have moved towards the reintroduction of semen stored at RT because selected progeny-tested sires can be used more efficiently in this way.

The relative advantages and disadvantages of frozen-thawed and liquid stored semen are reviewed by Vishwanath et al. (1996). Frozen semen made it possible to distribute sperm worldwide and reach areas where insemination with liquid semen is not practical (Foote and Parks, 1993). Furthermore, in a deep-frozen state, semen can be stored for years without any significant decrease in semen quality. Nevertheless, the cryopreservation process causes detrimental effects on bovine spermatozoa due to temperature reduction, cellular dehydration, freezing and thawing. This long-term preservation at low temperature causes irreversible damage to sperm membranes, killing a

large proportion of spermatozoa and rendering the remainder more sensitive to environmental stresses (Hammerstedt et al., 1990). Even under the best cryopreservation protocols approximately one-half of the initially motile population is irreversible damaged (Shannon and Vishwanath, 1995), causing a decrease in sperm lifespan, inability to interact with the female tract and a decrease in fertilizing ability. Shannon (1978) demonstrated that, in order to achieve comparable fertility rates with both fresh and frozen-thawed bull spermatozoa, 10 times the number of cryopreserved spermatozoa is required. Although the total number of spermatozoa used per insemination has approximately been halved over the intervening 20 years, the 10:1 ratio still applies; this means 10-15 million cryopreserved spermatozoa per insemination straw compared with 1-1.5 million fresh spermatozoa (Vishwanath et al., 1996). Another disadvantage of frozen-thawed semen is the requirement of a much higher investment in laboratory equipment while the necessary machinery and instruments incur heavy maintenance costs.

There has always been an interest in developing alternative strategies for long-term preservation of bovine spermatozoa. Experiments on desiccation, vitrification and freeze drying have been attempted in the past with limited success (Larson and Graham, 1976; Jeyendran et al., 1981; 1983). Other solutions are in vitro fertilization and, more recently, intracytoplasmic sperm injection (ICSI) into oocytes. However, these expensive methods are not useful in the bovine since they are not practical and do not solve the basic problems of poor sperm survival during cryopreservation (Holt, 1997).

Major gains could be made with liquid semen technology if the decline in fertility upon storage at RT or at 4°C is halted or reduced (Vishwanath and Shannon, 2000). Spermatozoa are essentially catabolic cells, so their post-ejaculation viability at normal body temperature is limited in vitro to a few hours for most species. Dilution of semen with a variety of physiological media can prolong the lifespan of spermatozoa by several hours in vitro. A better understanding of the mechanisms controlling sperm preservation in vivo may yield insights that could be important for storage of bovine spermatozoa in vitro. It is already known that the lifespan of ejaculated spermatozoa can be extended by placing them in co-culture with epithelial cells from the epididymis, oviduct and other types of somatic cells (Chian and Sirard, 1995). The mechanisms by which the presence of other cells supports spermatozoa are still unknown and differ for the various cell types.

Mammalian spermatozoa would probably be best stored in an immotile state. Some constituents of epididymal luminal plasma may be able to reversibly inhibit sperm motility and metabolism during storage in vitro. If this effect could be removed at the time of

insemination, such a procedure may allow storage of spermatozoa at RT or even at body temperature for extended periods. Future methods for preserving sperm fertility could also be directed at altering or reducing the catabolic metabolism of spermatozoa, increasing the resistance of sperm membranes to thermal and other environmental insults (Foote and Parks, 1993), and overcoming the effects of sperm ageing.

1.2 Aims of the study

The use of liquid-stored bovine semen, as an alternative to frozen storage would have obvious advantages in widespread selection schemes or in extensive production systems. Research is necessary to increase the time during which semen can be stored in liquid state whilst maintaining its fertilizing capacity. The fact that the cauda epididymidis has the capacity to store densely packed bovine spermatozoa in a potentially fertilizing condition for several weeks in vivo suggests that a study of the effects of epididymal constituents on spermatozoa may be useful for the formulation of better diluents for preserving spermatozoa at RT or at 4°C.

The general aims of the present thesis are to improve sperm storage in vitro and to develop alternative methods for the evaluation of sperm quality and bull fertility.

In order to achieve these aims, the study comprises the following experiments:

1. Examination of in vitro survival of bovine spermatozoa stored at room temperature under epididymal conditions.

2. Study of the effect of sperm coating on the survival and penetrating ability of in vitro stored bovine spermatozoa.

3. Comparison of the effect of hormones on protein secretion of caput and cauda epididymal epithelial cell culture in the bovine.

4. Investigation of the binding of spermatozoa from bulls with different non-return rates to epithelial oviduct explants by means of a new in vitro model.

5. Use of a sperm-oviduct binding assay for cooled bovine spermatozoa stored for several days in different diluents.

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2.

THE ROLE OF THE EPIDIDYMIS FOR SPERM CONSERVATION IN VIVO

Adapted from Vlaams Diergeneeskundig Tijdschrift 2000; 69: 159-167

De Pauw I., Van Soom A., Verberckmoes S. and de Kruif A.

2.1 Introduction

Spermatozoa are produced in the testis, outside the abdominal cavity in a pouch of skin called the scrotum. The scrotum protects the testicle against both extremes of temperature (Setchell, 1998). This is essential for normal sperm formation, which occurs only at a temperature several degrees below normal body temperature. Each testis is constituted of tubuli seminiferi containing spermatogonia, which are responsible for the production and the differentiation of spermatocytes into spermatids and finally into spermatozoa. This process takes several days and is called spermatogenesis. After formation, spermatozoa are transported into larger tubules to form the rete testis at the centre of the testis. Arising from the rete testis are 12 or more out-going ducts, the vasa efferentia, which emerge from the testis and enter the epididymis (Figure 1).

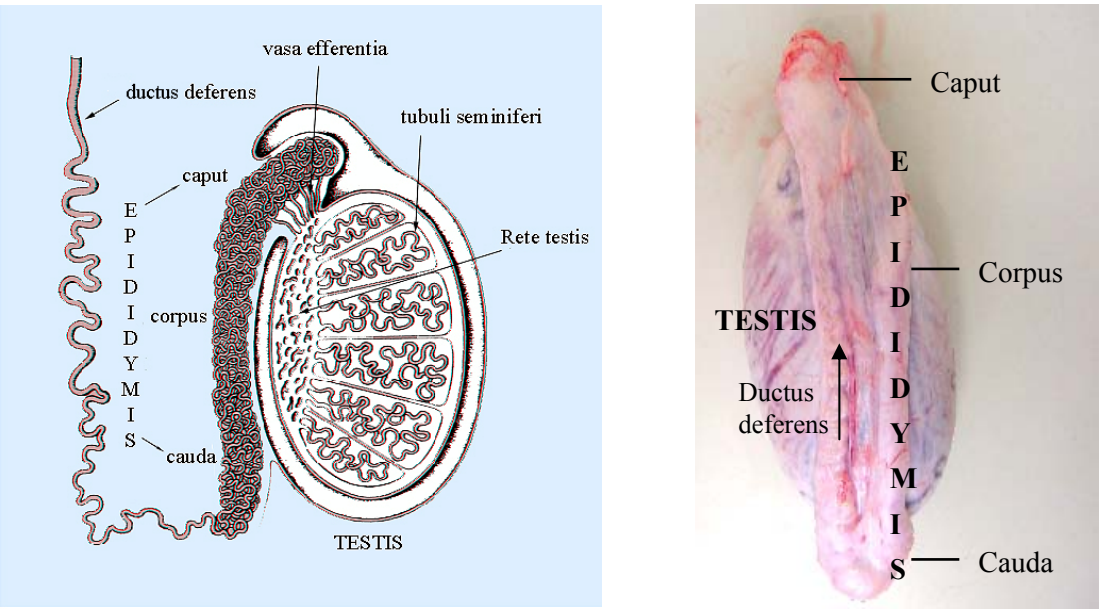

Figure 1: Testis and epididymis of the bull

The epididymis is a compact, flat, elongated structure closely attached to one side of the testicle. In the bovine it may cover a total length of 40 metres (Hoskins et al., 1978). The epididymis is anatomically divided into three major portions: caput (head), corpus (body) and cauda (tail) (Figure 1) and by histological criteria, several further subdivisions may be recognized (Cosentino and Cockett, 1986). Spermatozoa are transported further along the ductus deferentes. The ductus deferens is a muscular tube, which propels the spermatozoa from the cauda epididymidis to the urethra at the time of ejaculation. The accessory genital glands (seminal vesicles, prostate ands bulboutheral glands) are found in

the region where the left and right ductus deferentes unite into the urethra. These glands produce the secretions that make up most of the liquid portion of the semen of the bull. At ejaculation, the opening between the bladder and the beginning of the urethra closes. At the same time the spermatozoa and the accessory glands secretion mix together and enter the urethra to be forced out of the external opening at the apex of the penis. The volume of semen and the number of spermatozoa ejaculated by different bulls varies considerably. However, most bulls will ejaculate 3 to 5 ml of semen containing 1 billion spermatozoa per ml, or 3 to 5 billion spermatozoa per ejaculate.

Specific characteristics of spermatozoa confer to them the peculiarity of being a 'terminal cell'. The spermatozoon is a haploid cell, almost devoid of cytoplasm and other cellular organelles, except for the nucleus, the acrosome, and a series of mitochondria in an end-to-end helical arrangement located at the anterior region of the flagellum (Eddy and O'Brien, 1994). In the nucleus, the chromosomes are highly condensed and thus impede any transcriptional activity to replace proteins. The acrosome allows the spermatozoon to interact with and penetrate the oocyte at fertilization. The mitochondria provide ATP, which is mostly used to maintain motility. Spermatozoa lack the intracellular transport and storage systems of the endoplasmic reticulum and Golgi apparatus. To maintain functional cell membranes and to undergo maturational changes that confer to the sperm motility and ability to fertilize, sperm rely on the absorption of molecules from the surrounding environment (Amann et al., 1993; Yanagimachi, 1994).

2.2 Functions of the epididymis

The epididymis is an 'androgen target tissue', in which metabolism, epithelial secretion and maturation of the spermatozoa is regulated by androgens (Brooks, 1983; Robaire and Hermo, 1988). Androgen concentrations of the epididymis, especially of the caput are very high in comparison with the concentration present in serum (Pujol et al., 1976). Three major functions occur in the epididymis:

1. transport and maturation of spermatozoa
2. production of epididymal plasma
3. storage of mature spermatozoa in the cauda epididymidis until ejaculation

2.1.1 Transport and maturation of spermatozoa in the epididymis

Transport of spermatozoa in the epididymis

The time interval which spermatozoa require to pass along the entire length of the epididymal duct varies according to species, frequency of ejaculation and certain other conditions. The mean transit time of spermatozoa through caput and corpus epididymidis is rather consistent among most males of a species and is not influenced by frequency of ejaculation (Amann et al., 1976) (Table 1). Spermatozoa are transported through the cauda epididymidis in 3 - 13 days in most species (Amann et al., 1976; Robaire and Hermo, 1988). This time is dependent on the length of the epididymis and the storage capacity of the cauda. This period can be shortened by increasing the frequency of semen collection, but at the risk of depleting the reserve of spermatozoa in the cauda.

Table 1: Species differences in spermatozoal transit time through the epididymis (in days) (Amann et al., 1976)

Animal species	Caput	Corpus	Cauda
Bull	2.5	0.6	5.2
Stallion	1.9	2.2	9.8
Ram	2.4	1.2	12.8
Boar	2.2	3.2	6.4
Rat	2.1	0.8	9.8
Rabbit	2.2	0.8	9.7

Maturation of spermatozoa in the epididymis

Mammalian spermatozoa leaving the testis do not have the ability to recognize and bind to oocytes, nor to fuse with the oolemma (Moore and Akhondi, 1996). To acquire these abilities, they have to undergo numerous changes during epididymal transit. These include: morphological alterations (migration of cytoplasmic droplet down the tail), stabilization of nuclear chromatin by disulphide bonds, changes to the composition of membranes, increasing susceptibility to cold shock damage, development of motility and acquisition of fertility (Cooper, 1986; Bedford and Hoskins, 1990).

One of the most prominent changes in spermatozoa during epididymal maturation is the development of sperm's ability to move. This could be caused by biochemical and

physical changes of the sperm plasma membrane in combination with an increase of cAMP-concentration, a decrease of intracellular pH (pH_i), changes in free Ca^{2+}- ions and the velocity of glucose transportation in the spermatozoa (Hiipakka and Hammerstedt, 1978; Amann et al., 1993).

Spermatozoa are functionally mature in the cauda epididymidis but undergo additional membrane changes during ejaculation by components present in the seminal plasma. The process ends in the female reproductive tract, in which spermatozoa become capacitated.

2.1.2 Production of epididymal plasma

The most important functions of epididymal epithelium are the maintenance of a suitable environment for the maturation of spermatozoa in the caput and the storage of fertile spermatozoa in the cauda. More than 95% of the epididymal plasma is reabsorbed in the first part of the epididymis, leading to a sharp rise in spermatocrit (the volume of spermatozoa as a percentage of the volume of the plasma), and changes in the composition of the luminal fluid (Crabo, 1965). Between caput and cauda epididymidis, important changes occur in the composition of plasma (Crabo, 1965; Levine and Marsh, 1971; Setchell and Hinton, 1981). In fact, a large proportion of small molecules (amino acids, ions, sugars and water), large molecules (proteins and steroids) and other components of the testicular fluid are transported from the lumen of the epididymal duct to the interstitium of the epididymis. Some of the present components are produced by the concentrated epididymal spermatozoa themselves or reach the epididymis by blood or lymph.

2.1.3 Storage of mature spermatozoa in the cauda epididymidis

In mammals, the storage of spermatozoa occurs in the caudal part of the epididymis whereas in most reptiles spermatozoa are stored in the ductus deferens for many months (Licht, 1984). In most species, spermatozoa remain viable for several weeks (Table 2). The most extended sperm storage occurs in bats (10 months), owing to their hibernation (Gustafson, 1979). In birds, on the other hand, spermatozoa normally spend only about 24 h in the epididymis and ductus deferens before ejaculation (Clulow and Jones, 1982). Sperm production is almost continuous, although for some species seasonal variations occur (Setchell et al., 1993). In both cases, the number of spermatozoa present in the epididymis remains constant (Table 3). Approximately 55-65% of total epididymal

spermatozoa are stored in the cauda epididymidis (Amann et al., 1976) (Table 3). In the cauda of the epididymis, spermatozoa of different ages are present (Orgebin-Crist, 1965). This explains why individual spermatozoa from the same ejaculate capacitate and undergo the acrosome reaction at different time points (Cuasnicu and Bedford, 1989).

Table 2: Species differences in storage time of spermatozoa in the cauda epididymidis (days)

Animal species	Storage time in cauda (days)	Reference
Bull	30 - 45	Kirillov and Mozorov (1936)
Bat	10 months	Gustafson (1979)
Human	14 - 21 (or longer)	Turner (1995); Moore (1996)
Rat	50	Young (1931)
Guinea-pig	50	Young (1929)
Rabbit	30 - 60	Hammond and Asdell (1926)

Table 3: Species differences in the percentage of spermatozoa present in caput / corpus epididymidis, cauda epididymidis and ductus deferens

Species	# sperm (10^6)	Caput/Corpus (%)	Cauda (%)	Ductus deferens (%)	Reference
Bull	68.000	34	55	11	Amann et al. (1976)
Stallion	78.100	26	64	10	Amann et al. (1976)
Boar	191.000	46	54	-	Chang (1945)
Human	364	42	58	-	Amann (1981)
Rat	710	37	63	-	Breed (1986)
Hamster	1.210	13	84	3	Breed (1986)
Ram	131.500	19	79	1	Amann et al. (1976)
Rabbit	2.200	23	73	4	Amann et al. (1976)

Bovine spermatozoa are stored in a quiescent state in the cauda epididymidis prior to ejaculation (Cascieri at al., 1976; Carr and Acott, 1984). In this state, spermatozoa are immotile and metabolically inactive (Bishop and Walton, 1960), presumably to conserve energy stores required for their passage through the female reproductive tract and for the fertilization process (Mann and Lutwak-Mann, 1981; Zaneveld and Chatteron, 1982). Furthermore, this state leads to a decreased risk of alterations to membranes, internal structures and biochemical compounds by endogenous oxidizing agents produced by mitochondrial activity (Hamamah and Gatti, 1998).

This inhibition can be caused by the following factors:

1. low pH of the cauda epididymal plasma
2. hyperosmotic pressure
3. low oxygen concentration
4. high concentration of stored spermatozoa

These different storage conditions are summarized in table 4 and described in more detail in Chapter 4.

Table 4: Comparison of the different storage conditions of spermatozoa in the testis, cauda epididymidis and seminal plasma

	Testis	Cauda epididymal plasma	Seminal plasma
pH	7.3	6.7	6.7
Osmotic pressure	-	354 mOsm	300 mOsm
Storage atmosphere	low oxygen tension	low oxygen tension	aerobic
Sperm concentration	-	5 x 10^9/ml	0.5 x 10^9-1.5 x 10^9/ml
Temperature	33 - 35°C	33 - 35°C	38 - 39°C

In this quiescent state, bovine spermatozoa are stored for a relatively long period of time in which they retain their capacity for motility and fertilization much longer than samples kept in vitro at the same temperature or at RT (Jones and Murdoch, 1996). This immotile state can be of different length and ends when spermatozoa are released into seminal plasma at the time of ejaculation, when they become fully motile and metabolically active.

2.3 Biochemical composition of cauda epididymal plasma

Spermatozoa spend many days traversing the long epididymal duct and are constantly exposed to an ever-changing luminal fluid microenvironment, which is distinctly different in composition to that of blood plasma and interstitial fluid. This milieu is initially originating from the rete testis but becomes greatly modified by the secretory and absorptive activity of the epididymal epithelium (Setchell et al., 1993). Specific secretions from epididymal principal cells associate with spermatozoa during their maturation and storage (Moore, 1996) and play a fundamental role in modifying the surface characteristics of spermatozoa in preparation for the events of fertilization. Most investigations of the last years have focused on the role of the epididymis in sperm

maturation, however less research investments have been made in the understanding of sperm storage (Turner, 1995). The fact that the cauda epididymidis can store viable spermatozoa for several days in vivo suggests that some epididymal constituents could be very interesting in the formulation of diluents for preserving spermatozoa in vitro. Research on epididymal storage conditions can be performed by different approaches, such as the analysis of different physicochemical conditions or of the specific ionic and protein composition of the epididymal plasma, or by studying membrane differences between caput, corpus and cauda epididymal spermatozoa and ejaculated spermatozoa (Jones, 1998) or by examining in vitro interactions between spermatozoa and epididymal epithelial cells in culture (Moore, 1996). Chemical analysis of the composition of epididymal plasma of the bull has been investigated in different studies (Crabo, 1965; Cascieri et al., 1976; Setchell et al., 1993; Verberckmoes et al., in preparation). In our laboratory, analyses were performed with cauda epididymal plasma collected by making small incisions into the tubuli of post mortem material. The composition of this epididymal plasma was used to develop a new completely defined diluent named CEP-diluent (Verberckmoes et al., in preparation).

2.4 Conclusions

In mammals, spermatozoa leaving the testes are incapable of fertilizing a female gamete. Sperm maturation is the term given to the process by which mammalian spermatozoa undergo numerous changes during epididymal transit. The most important changes occurring in the spermatozoa are the development of motility and the acquisition of fertilizing ability, which are largely the result of interactions with epididymal epithelium and epididymal plasma. Spermatozoa spend many days traversing the long epididymal duct and they are finally stored in the cauda epididymidis in a quiescent state for several weeks awaiting ejaculation. In vivo, all these processes are coordinated with remarkable precision to ensure production of fully viable and fertile spermatozoa.

Although most investigations of the last decades have focused on the role of the epididymis in sperm maturation, it seems that research investment into the understanding of other major epididymal functions, especially sperm storage, should also be encouraged. There is practically no information on the effects of epididymal constituents on sperm survival and storage. In this respect, an interesting approach would probably be to search for a procedure that reduced sperm motility whilst maintaining its fertilizing capacity during storage but then allowed full development of motility when needed.

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3.

SUITABILITY OF SPERM FUNCTION TESTS FOR THE EVALUATION OF SPERM QUALITY AND BULL FERTILITY

Review in preparation

De Pauw I., Van Soom A., Verberckmoes S. and de Kruif A.

3.1 Introduction

Assessment of the functional capacity of spermatozoa in vitro is of particular interest in order to select sires with good sperm quality and fertility. Much research has already been done to use in vitro technologies to measure the fertilizing ability of spermatozoa. In many laboratories, the evaluation of semen is limited to the measurement of sperm concentration, microscopic examination of progressive motility and to the evaluation of morphology and membrane integrity by means of eosin/nigrosin staining. Methods for sperm analysis have considerably increased in recent years. It is now possible to replace subjective motility assessment by Computer Assisted Sperm Analysis (CASA) (Malmgren, 1997; Verstegen et al., 2002) and the former eosin/nigrosin staining method is now being superseded by fluorescent dyes that bind to various regions of the cell to demonstrate particular functional characteristics of spermatozoa (Harrison and Vickers, 1990; Johnson et al., 1996). Moreover, other technological changes over the past decade have made it possible to apply either new techniques such as flow cytometry (Garner et al., 1986; Graham et al., 1990) or more specific functional in vitro tests which determine sperm performance such as sperm migration (Kummerfeld et al., 1981; Anilkumar et al., 2001; Verberckmoes et al, 2002), sperm binding to oviduct epithelial explants (De Pauw et al., 2002) or to the zona pellucida (ZP) (Fazeli et al., 1997; Zhang et al., 1998) and in vitro fertilization (IVF) (Larsson and Rodriguez-Martinez, 2000) for the assessment of sperm quality and fertility. Several studies have tried to find a correlation between one or more sperm parameters and in vivo bull fertility (Table 1). Moreover, in some studies, a prediction of bull fertility was attempted by means of regression analysis, which tended to be more successful when multiple sperm parameters were taken into account. All studies had in common that in vivo bull fertility was assessed on a high number of inseminations, but for technical reasons it was difficult to include more than 20 bulls in this kind of research. Our aims were to discuss recent progress, which has been made in assessment of sperm quality in vitro, and to determine to what extent these relatively new tests, can be used for evaluation of in vivo bull fertility (Table 1).

3.2 The evaluation of bovine sperm quality characteristics by means of fluorescent stainings

Spermatozoa consist of several membrane compartments (i.e. plasma membrane, acrosomal membrane, mitochondrial membrane). Membrane integrity of all these compartments is of fundamental importance for the maintenance of the fertilizing potential of spermatozoa. Therefore, over the past 10-15 years many fluorescent stains have been developed that can be used to evaluate subcellular components indicating specific sperm functions (Amann, 1989; Oehninger et al., 1992; Critser and Noiles, 1993). The functional aspects that could give greater reliability to the estimation of the fertilizing capacity of spermatozoa which include plasma membrane integrity, capacitation, acrosome reaction, mitochondrial membrane potential and DNA integrity, are described below.

3.2.1 Membrane integrity

For many years, a number of procedures has been proposed to assess the membrane integrity of spermatozoa. The eosin/nigrosin staining (Figure 1A and B) has been used to assess both membrane integrity and morphology of the spermatozoa (Blom, 1950). This single association staining procedure is based on the degree of membrane permeability of dead spermatozoa in which heads take a pink (eosinophilic) colouration. It has been argued that this method is unreliable because, in some species, spermatozoa show partial staining (Hancock, 1957). Another disadvantage is that some ingredients such as glycerol present in commonly used cryopreservation media interfere with the staining (Mixner and Saroff, 1954).

In recent years several fluorescent probes have been developed to determine membrane integrity. The most used fluorophore is SYBR®-14 proposed by Garner et al. (1994). This fluorescent probe is used in combination with propidium iodide (PI). Both dyes have an affinity for nucleic acids. The membrane-permeant stain SYBR®-14 labels the DNA of all spermatozoa bright green, whereas PI has been used to detect only membrane-damaged spermatozoa (red fluorescence) (Garner et al., 1986). Upon cell death, spermatozoa lose their ability to resist the influx of the membrane-impermeant dye PI, which enters the spermatozoa through pores in the nuclear membrane that are located in the posterior region of the sperm head (Oko et al., 1976) and replaces or quenches the SYBR®-14 staining. Johnson et al. (1996) have discussed these cell colour changes in

terms of 'DNA viability' but in this instance the mode of action remains unclear (Holt, 2000). Normally there are three populations: living - SYBR®-14 stained (Figure 1C), dead - PI stained (Figure 1D), and a third population, which is double stained and represents dying spermatozoa (Figure 1E).

The advantages of this staining are its relative rapidity, accuracy and reliability. The staining time is not as critical as enzyme-based stains and background staining is virtually nonexistent (Garner and Johnson, 1995; Chalah and Brillard, 1998). The proportion of intact/damaged cells, sometimes called the live/dead ratio, can be estimated by either fluorescence microscopy or flow cytometry. Using this combination of dyes, membrane integrity of a sperm sample can be analysed with visible-light excitation, avoiding the harmful effects of ultraviolet (UV) exposure. Furthermore, Garner et al. (1996) have shown that fluorescent staining of boar spermatozoa with SYBR®-14 neither affected their ability to fertilize oocytes, nor the developmental competence of the resultant embryos.

Two other commonly used fluorescent stains are the bisbenzimide stains Hoechst 33342 and 33258, which excite in UV (365 nm) and require UV laser-equipped flow cytometry systems (Garner and Johnson, 1995). Also dual staining with enzyme-based staining combinations carboxyfluorescein diacetate and PI have been used, but the results are too variable for routine use (Garner and Johnson, 1995).

Comparisons of SYBR®-14/PI with eosin/nigrosin in sperm of different species have shown that the former stain is much more sensitive for detection of early membrane damage after centrifugation (dog: Rijsselaere et al., 2002) or after freeze-thawing (poultry: Chalah and Brillard, 1998; bull: De Pauw et al., 1999). It has been shown that post-thaw membrane integrity, as assessed by SYBR®-14/PI staining, is significantly correlated with field fertility. Furthermore, it is a better single predictor of fertility (r^2=0.39) than post-thaw motility, the proportion of capacitated spermatozoa and the proportion of induced acrosome-reacted spermatozoa (Januskauskas et al., 1999).

3.2.2 Capacitation

Although mammalian spermatozoa are actively motile after ejaculation, they are not yet able to fertilize eggs. To achieve this, they need to be activated in the female genital tract by two specific events called capacitation and acrosome reaction (Yanagimachi, 1994).

Capacitation can only be detected at the molecular level and not at the structural level. Several aspects of sperm capacitation have already been characterized including membrane and intracellular ionic modifications (de Lamirande et al., 1993; Yanagimachi, 1994; Visconti et al, 1998; Topper et al., 1999). These alternations involve removal or inactivation of decapacitation factors on the sperm surface; changes in localization, molecular structure and lateral mobility of membrane proteins; adsorption onto the sperm surface of proteins from the female tract (Yanagimachi, 1994); alterations in membrane lipid composition, in particular in the cholesterol/phospholipid ratio (Cross, 1998); ionic deregulation manifested as increases in internal Ca^{2+}, Na^{+} and pH (Fraser, 1995; Parrish et al., 1999); generation of reactive oxygen species (de Lamirande et al., 1997); and an increase in cAMP and protein tyrosine phosphorylation (Visconti et al., 1998). Capacitated spermatozoa are generally accepted to be fragile, unstable and short-lived (Hunter, 1987).

The fluorescent antibiotic chlortetracycline (CTC) has been utilized to assess the degree of destabilization of the sperm membrane (Fraser, 1995). This direct, single step staining assay is based on the transfer of neutral and uncomplexed CTC across the cell membranes into intracellular compartments containing high levels of free Ca^{2+}. Chlortetracycline binds to Ca^{2+} becoming more fluorescent as a result (Tsien, 1989). These CTC-Ca^{2+} complexes preferentially bind to hydrophobic regions of the cell membrane resulting in a pattern of membrane staining characteristics of various transitional phases of destabilization. Bovine spermatozoa stained with CTC display 3 different fluorescent patterns representing different stages of the capacitation process (Lee et al., 1987), which are indicated in literature by the letters F (uncapacitated, acrosome intact: Figure 1F), B (capacitated, acrosome intact: Figure 1G) and AR (acrosome reacted: Figure 1H). These patterns are quite difficult to distinguish by fluorescence microscopy at least in bull semen, and their classification is therefore rather subjective (De Pauw, unpublished observation). Moreover, the CTC staining method is found to be a laborious technique because it cannot be analyzed by flow cytometry (Rathi et al., 2001). The flow cytometric approach is based upon the intensity of the fluorescence of spermatozoa alone and lacks the precise delineation of specific fluorescence categories, which renders this method unsatisfactory for practical use (Maxwell and Johnson, 1997). Because of this disadvantage and the fact that the molecular basis of the interaction between CTC and the plasma membrane is still unclear, research is being undertaken to find alternatives for the evaluation of the capacitation status of spermatozoa.

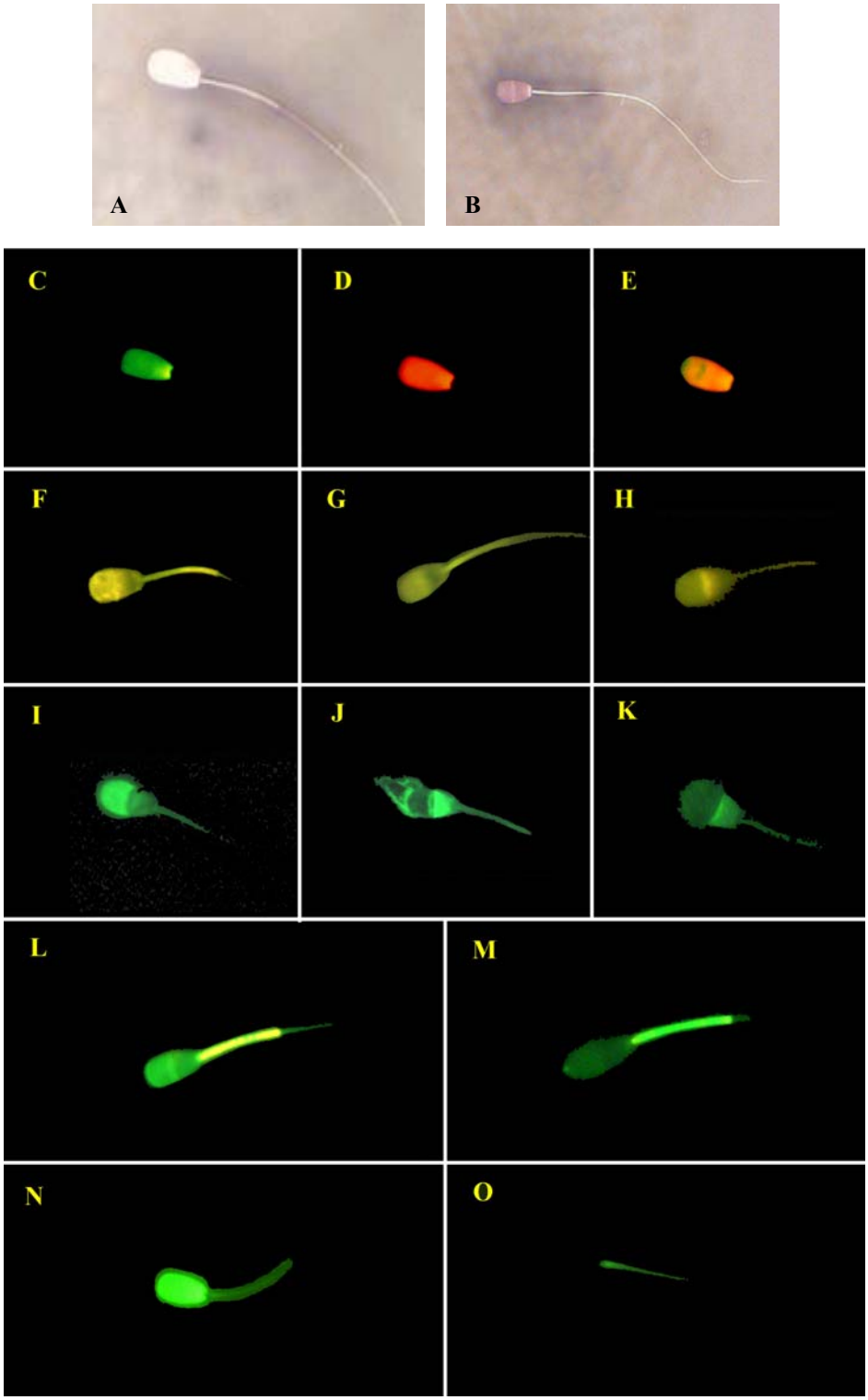

Figure 1: Fluorescent stains for the evaluation of subcellular components indicating specific sperm functions

Eosin (A)/**nigrosin** (B) and **SYBR-14** (C)/**PI** (D and E) staining for sperm membrane integrity; **CTC** staining for capacitation status of spermatozoa (F pattern (F), B pattern (G), AR pattern (H)); **PSA** staining for acrosomal status of spermatozoa (acrosome-intact (I) and acrosome-reacted (J and K)); **JC-1** staining for high (L) and low (M) mitochondrial membrane potential; **TUNEL** staining for DNA fragmentation (N) and DNA integrity (O) of spermatozoa.

According to Rathi et al. (2001), merocyanine 540 staining is a better method for the evaluation of the early events of capacitation. Merocyanine 540 is a hydrophobic dye that stains sperm membranes more intensely if their lipid components are in a higher state of disorder (Williamson et al., 1983; Langner et al., 1993). Another alternative could be filipin, a polyene antibiotic that forms 25-30 nm complexes with 3-ß-hydroxysterols. Filipin observes the cholesterol efflux in the sperm plasma membrane, one of the events during capacitation (Visconti et al., 1999). The distribution of filipin-sterol complexes can be visualized in freeze-fractured sperm membranes (Verkleij et al., 1973) or by UV-fluorescence due to the intrinsic fluorescent properties of filipin.

Nevertheless, CTC-staining has generally been accepted to be useful to evaluate the percentage of viable uncapacitated spermatozoa in frozen-thawed bull semen. The percentage of viable, uncapacitated spermatozoa in an AI-semen batch is positively correlated with its fertility after AI (Thundathil et al., 1999).

3.2.3 Acrosome reaction

Capacitated spermatozoa with ‘intact’ acrosomes may pass through the cumulus (Storey et al., 1984; Talbot, 1985), but are unable to pass through the ZP. The acrosome reaction, a modified form of exocytosis, is an absolute requirement for successful sperm passage through the ZP (Austin and Bishop, 1958). It involves the occurrence of point fusions between the outer acrosomal membrane and the overlying plasma membrane, followed by membrane vesiculation and extrusion of the acrosomal enzymatic contents, and exposure of inner acrosomal membrane antigens (Yanagimachi, 1994). The acrosomal content, containing mainly hydrolytic enzymes, starts to disperse and digest the ZP.

Different lectins interacting with glycoconjugates of the outer acrosomal membrane (Peanut agglutinin, PNA) or matrix (Pisum sativum agglutinin, PSA), have been tested for use in acrosomal status evaluation (Cross et al., 1986; Mortimer et al., 1987). Labeling permeabilized spermatozoa with fluorescein isothiocyanate-PSA (FITC-PSA) renders acrosome-intact cells brightly fluorescent over the entire acrosomal region of the sperm head (Figure 1I), while acrosome-reacted spermatozoa have either no acrosomal labeling, or only an equatorial band of label (Figure 1J and K). Peanut agglutinin binds to the anterior regions of the head. This binding is maybe more avidly than FITC-PSA, so that reacted spermatozoa with diminished FITC-PNA labeling are not as easily detected by visual inspection (Cross and Watson, 1994).

The integrity of the acrosome can also be determined morphologically, usually at the light microscopically level, in unstained samples or with different empirical stains such as Giemsa stain. However, fluorescent probes are generally superior to coloured dyes because fluorescence provides greater intensity and greater contrast between acrosomal and nonacrosomal material (Cross and Meizel, 1989). Moreover, FITC-PSA-staining has several other advantages over conventional stains: its simplicity, its objectiveness, its rapidity, its low cost, and more importantly many samples can be processed in parallel (Margalit et al., 1997).

The percentage of spermatozoa having an intact acrosome and being able to perform the acrosome reaction upon triggering is regarded as an important semen characteristic (de Leeuw et al., 1991). Acrosome reaction can be induced by treatment with calcium ionophores, and significant correlations have been found between the degree of induced AR and in vivo fertility of bulls, as assessed by non-return rates (Whitfield and Parkinson, 1995; Januskauskas et al., 2000).

3.2.4 Mitochondrial function

Mitochondria play an essential role in the life cycle of spermatozoa through the control of energy production in the form of adenosine triphosphate (ATP). The generation of ATP by mitochondria is vital to the survival of spermatozoa because it is the only source of ATP production and thus essential for cellular homeostasis (Moyes et al., 1998).

Mitochondrial membrane potential ($\Delta\psi_m$) is a sensitive indicator of the functional status of mitochondria and is used as a marker for assessing overall mitochondrial function (Cortopassi and Wong, 1999). Mitochondrial membrane potential is reduced when energy metabolism is disrupted, notably in apoptosis (Shapiro, 2000).

Several fluorescent stains such as Rhodamine 123 and Mitotracker Green selectively stain mitochondria in the midpiece of spermatozoa regardless of mitochondrial membrane potential. Another fluorescent stain, the lipophilic, cationic compound 5,5',6,6'-tetrachloro-1,1'13,3'-tetraethylbenzimidazolyl carbocyanine iodide (JC-1) can be used to evaluate mitochondrial function since it has been reported to differentially label mitochondria with high and low mitochondrial membrane potential (Reers et al., 1991; Cossarizza et al., 1993). JC-1 can form monomers in mitochondria with low membrane potential (green fluorescence; Figure 1M) and possesses the ability to form multimers known as J-aggregates after accumulation in mitochondria with high membrane potential

(orange fluorescence; Figure 1L). However, the intensity of JC-1 staining is dependent upon its concentration. This is one of the characteristics of JC-1 that makes it very difficult to get a proper interpretation of flow cytometric data. Furthermore, JC-1 also stains the head of the spermatozoa green. This staining at the membrane level contributes to the overall staining and makes its interpretation more difficult. Therefore, additional studies are needed to get a better understanding of the various aspects of the staining phenomena. It is obvious that JC-1 is a very interesting candidate molecule to include in sperm function testing. However, since no discernible relationship was found between mitochondrial membrane potential, measured with JC-1, and pre-freeze sperm motility (Garner et al., 1997), it may be useful to include more precise measurements of several motility characteristics obtained by CASA systems (Thomas et al., 1998). Until now, no studies are available linking JC-1 to in vivo bull fertility.

3.2.5 Apoptosis

Because spermatogenesis is a continuous process, a mechanism is needed to control sperm production. Apoptosis has been observed in male germ cells, which are phagocytosed by Sertoli cells in the testis (Xu et al., 1999). The failure of Sertoli cells to remove apoptotic spermatozoa results in their release into the lumen of seminiferous tubuli and, consequently, in an increased number of abnormal spermatozoa in semen, provoking reduced fertility (Anzar, 2002). Apoptosis, physiologically programmed cell death, affects single spermatozoon without any associated inflammation in the surrounding tissues (Wyllie et al., 1980). The process is characterized by a series of cellular, morphological and biochemical alternations in cells, including phosphatidylserine (PS) externalization, chromatin fragmentation and cell shrinkage (Wyllie et al., 1980; Earnshaw, 1995; Martin et al., 1995).

To measure apoptosis in spermatozoa two flow cytometric methods can be used. The first method is based on an assay for the measurement of PS translocation across the plasma membrane. Phosphatidylserine, normally present on the inner leaflet of the plasma membrane of healthy cells, is translocated and exposed on the outer leaflet during early apoptosis (Martin et al., 1995). Annexin V is a Ca^{2+}-dependent, phospholipid-binding protein (35-36 kDa) with a high affinity for PS (Vermes et al., 1995) and conjugated to fluorescein isothiocyanate (FITC) fluorochrome it can serve as a sensitive probe that can be used for flow cytometric detection of apoptosis. According to Anzar et al. (2002), the

Annexin V/PI assay is more precise and reliable than the common live/dead SYBR®-14/PI assay because the staining is sensitive to alterations in the sperm plasma membrane at the molecular level. SYBR®-14 stains apoptotic as well as live spermatozoa in a population, because in both cases the plasma membrane is intact. In contrast, Annexin V/PI assay is able to distinguish between apoptotic and live spermatozoa.

The second method is an assay for nicked DNA using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) and is also based on flow cytometry. The activation of an endogenous endonuclease that generates numerous DNA-strand breaks in chromatin is the most characteristic feature of apoptosis occurring relatively late in the apoptosis cascade. The TUNEL assay is specific for double-strand breaks and does not detect single-stranded nicks (Didier et al., 1996). Although the TUNEL assay can be used to evaluate a single spermatozoon, there are limitations to the sensitivity of this technique, and many spermatozoa with damaged DNA might escape detection (Sakkas et al., 1999). TUNEL staining was observed after 6 h while the detection of PS translocation using Annexin V staining was already observed after 1 h (Godard et al., 1999). According to Shen et al. (2002), Annexin V staining appears to be more sensitive than the TUNEL assay for the detection of apoptotic spermatozoa. Using both assays, apoptotic spermatozoa show green fluorescence (Figure 1N) while fully viable spermatozoa are non-fluorescent (Figure 1O). No correlation was found between the relative number of spermatozoa that expresses PS and the number of spermatozoa that show DNA-strand breaks (Oosterhuis et al., 2000).

In fresh semen, the number of apoptotic spermatozoa varies among bulls but is generally below 17% of the total number of spermatozoa. However, sperm preservation can also induce apoptosis. It has been shown that after freezing and thawing bull semen, the apoptotic spermatozoa accounted for more than 31% in the sperm samples (Anzar et al., 2002). The same researchers also found that bull fertility was negatively correlated with the percentage apoptotic fresh spermatozoa possessing nicked DNA as detected by the TUNEL assay, and positively correlated with the percentage viable fresh spermatozoa as detected by the Annexin V/PI assay.

3.3 Sperm motility assessment

Flagellar motion is a fundamental expression of sperm quality and is essential for its reproductive function. Following insemination, mammalian spermatozoa exhibit a

sequence of different types of motility modulated by the female reproductive tract. Vigorous motility is not only necessary for successful sperm migration to the site of fertilization but also for the transit through the cumulus cell investments and the zona pellucida of the oocyte.

Motility evaluation is based upon subjective estimates of the percentage of spermatozoa exhibiting any kind of movement and the proportion of spermatozoa moving progressively forward. This assessment is mainly based on subjective optical microscopic evaluation which is cheap and easy to perform. However, sperm motility estimates vary among examiners even when examing the same sperm sample. These variations are not only dependent on the biological state of the spermatozoa but also on the conditions of observation (Drobnis and Katz, 1990), such as the physical properties of the suspending fluid, the depth of the observation chamber and the observers' training.

Subjective evaluation of sperm progressive motility is used to determine the quality of bull semen intended for AI. However, subjectively assessed motility is not a good predictor of the fertility level of the AI-semen dose (Soderquist et al., 1991), particularly when motility values are within ranges around 50% (Stalhammar et al., 1994). This was confirmed by den Daas (1997) who concluded that the measurement of motility characteristics of serially diluted sperm, after thawing or after thawing followed by a thermo-resistance or endurance test, cannot be used to predict individual bull fertility.

Measurement of sperm motility is entering a new era in which objective, efficient and rapid methods are becoming accessible. Nowadays sperm motility can be assessed by computerized measurements, but also by approaching motility in a more functional way: by analyzing sperm migration through a viscous fluid or by evaluating the percentage of spermatozoa displaying chemotaxis.

3.3.1 Motility assessment by computer assisted sperm analysis (CASA) systems

Due to the variation between subjective estimates of sperm motility, more emphasis has been put upon the development of objective methods for quantifying sperm motion. Time-lapse photomicrography, multiple-exposure photomicrography, and frame playback video-micrography have been used (Malmgren, 1997). However these methods are labor-intensive. One recent development is the application of computerized image analysis systems, which offer an automated, rapid and objective approach of more specific characteristics of spermatozoa motion, such as straight-line velocity (VSL), curvilinear

velocity (VCL), average path velocity (VAP), linearity (LIN), straightness (STR), circularly motile spermatozoa (CIR), lateral head displacement (LHD), and beat cross frequency (BCF). The main problems are the high cost of the equipment, and standardization and optimization of the system and procedures (Verstegen, 2002). Before any practical use is possible, careful validation and checking of the setup are very important since using a wrong setup might lead to a wrong estimation of motility. Computerized systems can provide precise and accurate information on sperm motion characteristics for different species. However a lack of uniformity among users and instruments makes it difficult to define standard accepted values for normal and abnormal sperm motion (Verstegen, 2002).

It is still not clear which sperm movement characteristic will be of clinical value for the prediction of in vivo fertility and fertility rates (Aitken et al., 1985; Amann, 1989). Farrell et al. (1998) have found that the combination of percentages of motility and velocity was highly correlated with fertility (r^2=0.87), despite the fact that bulls differ largely in all variables related to motility and velocity mainly caused by the large interanimal variation.

3.3.2 Motility assessment by sperm migration assays

Spermatozoa are selected both quantitatively and qualitatively during their migration through the cervical mucus and during their transit in the female genital tract (Jouannet and Feneux, 1987). Cervical mucus is a physiological barrier controlling sperm access to the upper female reproductive tract. Spermatozoa with anomalies of the flagellum and particularly of the middle piece are not able to migrate through the cervical mucus (Jeulin et al., 1985). Furthermore, the pattern of sperm movement and amplitude of the lateral head displacement are also very important for cervical mucus penetration (Aitken et al., 1985).

In human, sperm migration tests have been extensively refined and validated (Biljan et al., 1994; Clarke et al., 1998). In contrast to human (Alexander, 1981), no correlation has been found between bull fertilizing capacity of spermatozoa and sperm migration capacity (Kummerfeld et al., 1981; Verberckmoes et al., 2002). There are several explanations for this lack of correlation (Verberckmoes et al., 2002). First, it could be due to the small difference in fertility between bulls. Sperm migration tests are performed with spermatozoa of bulls used for AI; whereas in human, it is possible to

compare spermatozoa of fertile donors with spermatozoa of patients with infertility problems. Another explanation could be the fact that NRRs are determined after AI. In this case spermatozoa don't have to migrate through the cervical mucus as they are deposited directly in the uterine body or in the uterine horns.

3.3.3 Motility assessment by sperm chemotaxis assays

A unique feature of sperm chemotaxis is the directional change of movement towards the source of an attractant or retreat from a repellent. The most commonly used technique for studying sperm chemotaxis in mammals is an 'accumulation assay' in which spermatozoa sense an ascending gradient of the attractant and accumulate near or at its source. Another commonly used assay is a 'choice assay' in which spermatozoa choose between two wells: one containing the attractant and the other containing the buffer as a control (Eisenbach, 1999).

Sperm chemotaxis to follicular fluid has already been established both in human (Cohen-Dayag et al., 1994; Ralt et al., 1994) and mouse (Giojalas and Rovasio, 1998; Oliviera et al., 1999). However, the fraction of chemotactic spermatozoa in the total sperm population is very small (2-12% in human (Cohen-Dayag et al., 1994) and approximately 10% in mouse (Giojalas and Rovasio, 1998; Oliviera et al., 1999)). According to Makler et al. (1992, 1995), chemotaxis between human sperm and ova does not exist because the fraction of these responsive spermatozoa is too small, the chemotactic responsiveness is only temporary and many 'chemotaxis assays' have failed to distinguish between chemotaxis and other accumulation-causing processes.

Many other reasons prevented the acquisition of conclusive evidence to the concept of mammalian sperm chemotaxis. In mammals, many ejaculated spermatozoa are able to reach the egg by chance. False results could be obtained because the signal-to-noise ratio in the measurements is very low (Cohen-Dayag et al., 1994; Oliveira et al., 1999), the variability between samples is too large or the used attractant concentration is too high (Eisenbach, 1999). Furthermore, mammalian sperm attractants have not been identified and the molecular mechanism of sperm chemotaxis is still unknown. Until now, only one in vitro study has been published which is indicative for a possible chemo-attractive role of the cumulus cells during bovine fertilization (Chian et al., 1996).

In our laboratory attempts were made to investigate sperm chemotaxis to cumulus-oocytes complexes (COC) and to medium conditioned by mature COC by means of a

choice assay in bovine. However, no conclusions could be made since it is rather difficult to count the low concentration of chemotactic spermatozoa and to make a functional migration assay without interference of physical forces. More research is therefore needed to refine comparative assays to test for chemotactic substances secreted by cumulus cells (Van Soom et al., 2002).

3.4 Sperm binding and performance assessment

Spermatozoa have to conquer several barriers in the female tract, which selects motile and morphologically normal spermatozoa to reach the site of fertilization (Figure 2). Depending on the species and the site of sperm deposition, fertilizing spermatozoa are stored in the isthmus 15 min to 8 h after insemination, and will remain attached to oviduct epithelial cells until ovulation (Hunter, 1996; Smith, 1998; Suarez, 1998; Scott, 2000). Upon ovulation capacitated spermatozoa are released from the oviduct epithelial cells and swim up to the oocyte. In some cases they have to pass through the cumulus oophorus to bind to the ZP and start the acrosome reaction required to penetrate the ZP and to fuse with the oolemma. Spermatozoa should be able to perform all these functions and therefore function tests could be useful to find a correlation with in vivo bull fertility (Larsson and Rodriguez-Martinez, 2000).

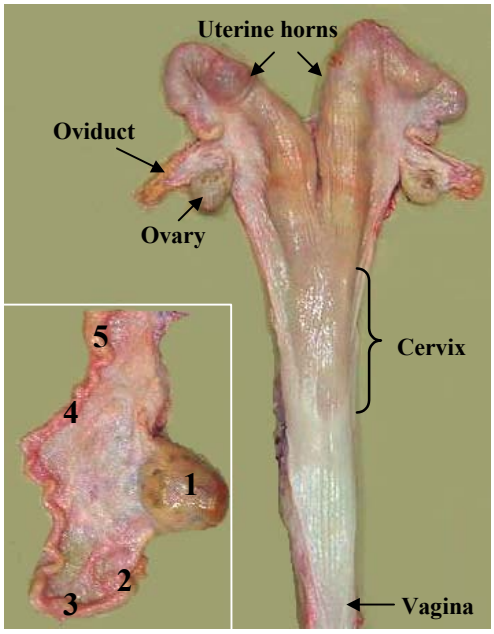

Figure 2: The female reproductive tract of the cow. The inset illustrates the oviduct and ovary at a higher magnification (1. ovary; 2. infundibulum of the oviduct; 3. ampulla of the oviduct (site of fertilization); 4. isthmus of the oviduct (site of sperm capacitation); 5. uterine horn).

3.4.1 Sperm-oviduct binding

In most mammalian species, except for humans, a considerable fraction of ejaculated spermatozoa is retained with reduced motility in the lower segment of the oviduct. The high mucus-containing narrow lumen of the oviductal isthmus impedes their forward progression. Spermatozoa bind strongly to carbohydrate moieties on glycoproteins or glycolipids on the surface of the oviductal epithelium and are stored there (Suarez, 1998). In cattle, it takes about 6 to 8 hours before a sufficient number of spermatozoa has reached this sperm reservoir to ensure fertilization (Hunter and Wilmut, 1982). The period from the onset of oestrus to the completion of ovulation may be as long as 30 h, therefore the population of spermatozoa capable of fertilization may spend up to 22 h in the isthmus. When ovulation occurs, some spermatozoa in this reservoir resume high motility and travel the distance between this storage site and the fertilization site at the oviductal ampulla within minutes (Hunter, 1993). It is obvious that when a bull is unable to populate the sperm reservoir with a sufficient number of spermatozoa, this might interfere with its fertility. It is likely that differences exist among bulls in their capacity to establish a sperm reservoir after mating or insemination. However, it is difficult to detect these differences after matings and collection of oviducts, because the number of spermatozoa reported to reach the oviduct in vivo varied considerably between studies and within experiments (Parker et al., 1975; Mburu et al., 1996; Suarez et al., 1997). Our laboratory has optimized an in vitro approach to study sperm binding to oviduct explants, which can be used to determine whether the capacity to establish a reservoir is indicative of fertility (De Pauw et al., 2002). By means of this in vitro model it has been shown that the capacity of spermatozoa to bind to oviduct explants in vitro varies among bulls and evidence was obtained that sperm binding to oviduct explants is related to in vivo fertility of the donor. Further refinements to this sperm-oviduct binding assay could be made when the molecular interaction of this sperm function is better understood. In this respect, measurement of the expression of sperm surface adhesion molecules by means of antibodies or specific ligands would be an option. Levels of fucose-binding protein, which can be detected with a fucose-BSA-FITC conjugate, on the surface of uncapacitated spermatozoa (Ignotz et al., 2001) may be associated with the capacity of a given sperm sample to bind to oviduct epithelial cells.

3.4.2 Sperm-zona binding

Before a spermatozoon penetrates the oocyte, it binds selectively to the homologous ZP, which is an important preliminary step in the fertilization process (Gould et al., 1983). The ability of spermatozoa to bind to the ZP reflects multiple functions such as membrane integrity, motility, morphology, acrosomal status and the ability to penetrate oocyte investments (Burkman et al., 1988; Kaskar et al., 1994; Liu and Baker, 1994).

Two types of sperm-ZP binding assays have been tested for bull spermatozoa: one using intact (not cleaved) homologous oocytes (zona binding assay, ZBA) (Fazeli et al., 1993; Zhang et al, 1995), the other using bisected hemizonae (hemizona binding assay, HZA) (Fazeli et al., 1997; Franken et al., 1997). The latter implies a micro-bisection of the oocytes into two matching hemizonas; each of one is incubated with spermatozoa from a test bull and a fertile control bull, respectively (Franken et al., 1997).

In human, it has been shown that homologous test systems based on sperm-ZP binding can be used to predict the outcome of IVF (Burkman et al., 1988; Liu et al., 1988). In contrast, no significant relation between the ZBA and AI-fertility of bull semen samples was found (Fazeli et al., 1997; Zhang et al., 1998). These assays have shown large intra- and interassay variations in the amount of sperm binding, probably as a consequence of the quality or maturational status of the oocytes (Mahadevan et al., 1987; Familiari et al., 1988, Oehninger et al., 1989). However, significant correlations with AI-fertility were only found when the ZBA was performed with an increased number of intact oocytes and testing a large population of frozen semen batches from AI bulls (Fazeli et al., 1997, Zhang et al., 1998). A significant relationship was also found between HZA and in vivo fertility of bull and stallion spermatozoa (Fazeli et al., 1995; Zhang et al., 1998).

An interesting alternative could be the use of commercially available synthetic beads coated with recombinant ZP proteins. Preliminary experiments by Whitmarsh et al. (1996) have revealed significantly higher levels of sperm binding to rhuZP3 beads compared with controls. Further refinements are needed so that a solid-phase sperm function test based on rhuZP3-coated beads is likely to be developed in the near future. A modification to HZA has been reported whereby acrosomal dyes have been incorporated to provide the indication of the acrosomal status of the bound spermatozoa. Zona pellucida penetration has also been described as an adjunct to the basic assay (De Jonge, 1999).

3.4.3 In vitro fertilization

In vitro fertility can be assessed by penetration tests (Wheeler and Seidel, 1987; Tatemoto et al., 1994; Matás et al., 1996; Hay et al., 1997; Gadea et al., 1998). For penetration of the oocyte in vitro spermatozoa need to be motile, capacitated, and able to bind to the ZP, to acrosome react, to penetrate the ZP, to fuse with the oocyte and be able to show decondensation of the nucleus. The oocyte penetration rate is measured as the number of oocytes with a visible (decondensed) sperm head in the cytoplasm of the oocyte 18 h after onset of fertilization. The limitation of this penetration test is the variability of oocyte maturation between different oocyte batches, which adds substantially to the variation between tests. Therefore evaluation can only be performed by comparing results of bulls on the same group of oocytes (den Daas, 1997). Bulls were found to vary in the total number of oocytes penetrated as well as in the increase of the penetration rate after raising the sperm oocyte ratio. However, oocyte penetration rate in vitro cannot be used for the prediction of fertility results after insemination (den Daas, 1997). This could be explained by differences between the in vivo and in vitro situation. Firstly, an undifferentiated population of spermatozoa is presented in vitro whereas in vivo, only a selected population of spermatozoa is able to reach the fertilization site. Secondly, the ovulated bovine oocyte encounters only a few free-swimming spermatozoa at the site of fertilization (sperm:oocyte ratio is close to 1:1) (Hunter, 1993). In most IVF systems, sperm concentration ranges from 0.5 x 10^6 to 5 x 10^6 spermatozoa/ml (Gordon, 1994). Finally, in vivo the oocyte is denuded during or shortly after ovulation in contrast to most in vitro systems where spermatozoa have to traverse the COC to reach the ZP (Ward et al., 2002). A recent study elucidated marked differences in the kinetics of sperm penetration between sires of different in vitro and in vivo fertility (Ward et al., 2002). Faster penetrating bulls were ranked higher in field fertility. After only 3 h of gamete co-incubation a correlation coefficient of 0.827 was found between NRR and oocytes penetration whereas after 12 h the correlation was only 0.67 and not significant.

A different approach for testing bull spermatozoa in a zona-free oocyte penetration test was described by Henault et al. (1995). Spermatozoa from bulls differing in in vivo fertility were labeled with fluorescent dyes with colour patterns specific for a given bull, in order to assess their relative abilities to penetrate the same zona-free oocytes. The results showed that spermatozoa from highly fertile bulls were superior to spermatozoa from low-fertile bulls. The authors concluded that this penetration assay is a reliable method for

detecting differences in relative bull fertility.

The percentage of oocytes developing to the blastocyst stage is a better measure of fertility than the percentage of oocytes penetrated by spermatozoa because two-thirds of oocytes fail to reach the blastocyst stage (Gordon, 1994) and would not result in pregnancy. The endpoints are cleaved oocytes and/or their development to morulae or blastocysts.

The correlation between IVF and fertility after AI using bull semen has been studied showing a positive correlation in some studies (Marquant-LeGuienne et al., 1990, 1992; Shamsuddin and Larsson, 1993; Zhang et al., 1997) while no correlation in others (Schneider et al., 1999). Ward et al. (2001) have shown that kinetics of early cleavage can be used to distinguish between bulls of high and low fertility. They observed the strongest correlation at 33 hpi. This is a perfect example of how sufficient refining and running a test can yield significant correlations between in vitro tests and in vivo fertility. It can serve as a model for adaptations of other function tests for practical applications.

However, being an expensive and time-consuming procedure, IVF cannot be routinely used as a screening test. Furthermore, if fertilization fails the problem could be in any of the preceding steps or may actually be due to the oocytes rather than to the spermatozoa.

3.5 Conclusions

Several laboratory assays have been developed with the aim of directly analyzing fertilization processes. However, the complexity of the fertilization process makes individual assays far less than optimal. The combination of several semen quality measurements should increase the accuracy of fertility estimates. Combinations of fluorescent stains can be used to simultaneously assess several different functional characteristics of spermatozoa, thereby increasing the likelihood of reflecting their fertilizing capability. However, results obtained from different evaluation techniques can be contradictory, and consequently misinterpreted. This makes routine semen analysis not a valid predictor of the ejaculate's fertilizing ability.

Functional tests, which provide valuable knowledge about selection of spermatozoa along their route to the oocyte or about sperm-oocyte interaction, undergo continual development in an effort to accurately estimate fertility. However, until now most of these complementary tests are only able to exclude the most subfertile bulls. Also confusion and

disagreement still remains concerning the usefulness of some assays and the correct interpretation of their results.

Refinement, standardization and validation of sperm function assays are therefore very important to improve their predictive capacity. A better understanding and further clarification of the complex processes of capacitation, acrosome reaction, sperm-oviduct binding and sperm-oocyte interactions could lead to the identification of specific molecules important in these sperm functions.

3.6 References

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4.

IN VITRO SURVIVAL OF BOVINE SPERMATOZOA STORED AT ROOM TEMPERATURE UNDER EPIDIDYMAL CONDITIONS

Theriogenology 2003; 59: 1093-1107

De Pauw I., Van Soom A., Mintiens K., Verberckmoes S. and de Kruif A.

Abstract

In this study, environmental conditions mimicking those prevailing in the epididymis were used for storing ejaculated bull spermatozoa in vitro during 4 days at room temperature (RT). These conditions were low pH, high osmolarity, high sperm concentration and low oxygen tension. Hepes-TALP was used as basic storage medium. Ejaculated spermatozoa were stored at a concentration of 10 x 10^6 spermatozoa/ml in Hepes-TALP of different pH (4, 5, 6, 7 or 8) and osmolarity (100, 300, 400, 500, 600 or 800 mOsm) and under different atmospheric conditions (nitrogen-gassed or aerobic). Spermatozoa were also stored undiluted or at different concentrations: 10 x 10^6, 100 x 10^6, 500 x 10^6 or 1 x 10^9 spermatozoa/ml. Sperm parameters such as membrane integrity, motility, mitochondrial membrane potential or DNA fragmentation were used to assess semen quality after storage. Adjustment of the pH of Hepes-TALP to pH 6 yielded significantly better results than storage at all other pH values. Isotonic Hepes-TALP had a less detrimental effect on spermatozoa than the hypo- and hyperosmotic versions. No differences in sperm parameters were observed when spermatozoa were incubated under aerobic or under nitrogen-gassed storage conditions. Optimal sperm concentration in vitro is 10 x 10^6 spermatozoa/ml. This is in contrast with the in vivo situation, where spermatozoa are stored at a high concentration. Better results at high sperm concentrations were obtained when spermatozoa were diluted for less than 5 minutes in Triladyl®-diluent supplemented with egg yolk and glycerol immediately after ejaculation.

Introduction

Ejaculated spermatozoa diluted in vitro remains viable for a limited period of time. Much research has been done, especially for bovine semen, to understand the effect of different factors and to design a useful storage medium to enhance sperm survival in a non-frozen state. Despite the development of numerous diluents (reviewed by Vishwanath and Shannon, 2000) the fertile lifespan of spermatozoa is maintained for only 3-5 days when stored in a liquid environment at high dilutions at ambient temperatures (Vishwanath and Shannon, 1997). In contrast to their limited lifespan in vitro, spermatozoa can remain viable for several weeks when stored in the cauda of the epididymis (Setchell et al., 1993). During epididymal transit, mammalian spermatozoa acquire the capacity for motility and for fertilization. When they reach the caudal part of the epididymis, they must be maintained in a quiescent state awaiting ejaculation, presumably to conserve energy required for the fertilization process (Mann and Lutwak-Mann, 1981; Zaneveld and Chatterton, 1982). In this quiescent state, bovine spermatozoa are immotile and metabolically inactive (Cascieri et al., 1976). Spermatozoa are stored for a relatively long period in this state, during which they retain their capacity for motility and the fertilization process much longer than samples kept in vitro at the same temperature (Jones and Murdoch, 1996). It is obvious that the environment in the cauda epididymidis favours the lifespan of the spermatozoa. The cauda epididymidis is characterized by low pH (Acott and Carr, 1984; Carr et al., 1985) and hyperosmotic pressure of the plasma (Liu and Foote, 1998), spermatozoa are stored at high concentrations (Cascieri et al., 1976; Amann et al., 1976) and at low oxygen tension (Free et al., 1976).

Until now it has been very difficult to mimic these epididymal conditions in vitro, although some reports have shown that spermatozoa can exhibit some fertilizing ability after being stored for a few days in cooled epididymides of different species (Kikuchi et al., 1998; Browne et al., 2001; Sankai et al., 2001). Unfortunately, this is not a practical alternative for sperm storage, so we have chosen to investigate the influence of different epididymal conditions upon sperm survival in diluent at RT. Since it is known that sperm fertility declines much faster than sperm motility (Vishwanath and Shannon, 1997, 2000), less conventional sperm characteristics were also examined such as mitochondrial membrane potential and the prevalence of DNA fragmentation in stored spermatozoa. Fragmentation of genomic DNA can be detected by means of terminal deoxynucleotid transferase mediated dUTP nick end labeling (TUNEL), and mitochondrial membrane

potential, which is related to the presence of a low mitochondrial transmembrane potential which can be assessed by 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) staining. These characteristics of apoptosis might be related to sperm fertility, as it has been shown in human semen samples (Sun et al., 1997; Donnelly et al., 2000).

In our study, the simple salt solution Hepes-TALP was used as the principal storage medium. The effect of pH, osmolarity, storage atmosphere and sperm concentration on membrane integrity, motility and mitochondrial function of bull spermatozoa stored for 3 or 4 days at RT was examined. Sperm nuclear DNA fragmentation was determined in order to quantify possible apoptosis, which might occur during prolonged storage of spermatozoa at RT. The sperm collection method was adapted in order to "coat" spermatozoa with egg yolk-glycerol to improve subsequent sperm survival.

Materials and Methods

Collection and preparation of semen

Semen was collected from a 2-year-old Red Pied bull using an artificial vagina at 1-week intervals. Immediately after collection, spermatozoa were separated from the seminal plasma by centrifugation (720 x g, 10 min) at RT. The sperm concentration was determined using a Bürker chamber and subsequently diluted to a concentration of 10×10^6 spermatozoa/ml in Hepes-buffered TALP media of different pH or osmolarity, except for the experiment where the effect of sperm concentration itself was examined. Spermatozoa were stored at RT in darkness under different incubation conditions according to the experiment (72 or 96 h). After storage of the spermatozoa under the different conditions, samples were taken and evaluated microscopically or by flow cytometric analysis for functional parameters such as membrane integrity, motility, mitochondrial membrane potential, morphology and DNA fragmentation.

Media

Chemicals and media were obtained from Sigma (Bornem, Belgium) and LifeTechnologies, Gibco BRL® products (Merelbeke, Belgium).

The principal storage medium was Hepes-TALP containing 114 mM NaCl, 3.1 mM KCl, 0.3 mM NaH_2PO_4, 2.1 mM $CaCl_2$, 0.4 mM $MgCl_2$, 2 mM $NaHCO_3$, 0.2 mM sodium pyruvate, 10 mM sodium lactate, 10 µg/ml gentamycin sulphate, 10 mM Hepes and 3 mg/ml Bovine Serum Albumin. The pH and the osmolarity of this basic medium were modified according to the experiments.

Processing of sperm samples

The percentage of motile spermatozoa was subjectively assessed by visual estimation of two investigators. To achieve this, a small droplet of spermatozoa was placed on a glass slide, covered with a glass cover slip, and viewed under a light microscope (magnification 200 x) equipped with a stage warmer (37°C).

Fluorescent staining techniques were used for the evaluation of membrane integrity and mitochondrial membrane potential. The nucleic stains SYBR®-14 and propidium iodide (PI) were used for analyzing membrane integrity of the spermatozoa (LIVE/DEAD® Sperm Viability Kit (Molecular Probes, Leiden, The Netherlands)). A working solution of 1 mM SYBR®-14 dye reagent in 100 % anhydrous dimethyl sulfoxide (DMSO) was first diluted 1:50 in Hepes-buffered medium and added to 1 ml of the diluted sperm sample. The DNA of membrane-intact spermatozoa was labeled with SYBR®-14, resulting in a bright green fluorescence when excited at 488 nm. Propidium iodide dissolved in water to a concentration of 2.4 mM stained the nuclei of membrane-damaged spermatozoa red (excitation: 530 nm). The combination of these two fluorescent stains makes it possible to distinguish both membrane-intact and membrane-damaged spermatozoa within the same preparations. Both nucleic stains SYBR®-14 and PI work effectively as a live/dead stain for either fluorescence microscopy or flow cytometry.

For evaluation of mitochondrial membrane potential, 2 µl of 1.53 mM JC-1 (Molecular Probes, Leiden, The Netherlands) in DMSO was added to 1 ml of a diluted semen sample in combination with 0.7 µl SYBR®-14 and 12 µl of 2.99 mM PI (Thomas et al., 1998; Garner and Thomas, 1999).

Fluorescence analyses of stained spermatozoa

Sperm membrane integrity and mitochondrial membrane potential were evaluated using fluorescence microscopy and flow cytometry.

Fluorescence microscopic analysis was performed using a Leica DMR microscope (Van Hopplynus NV, Brussels, Belgium) equipped with an excitation filter of 450 - 490 nm from a 100 W mercury lamp and examined at magnification 1000 x.

Sperm samples labeled with SYBR®-14/PI displayed three types of fluorescent spermatozoa: membrane-damaged spermatozoa with red heads, membrane-intact spermatozoa with green heads and moribund spermatozoa, which were dual stained.

Sperm samples labeled with JC-1 and SYBR®-14/PI displayed three types of fluorescent spermatozoa: those with red heads and green mitochondria (membrane-damaged spermatozoa), those with green heads and orange mitochondria (membrane-intact spermatozoa with high mitochondrial membrane potential) and those with green heads and green mitochondria (membrane-intact spermatozoa with low mitochondrial membrane potential).

Flow cytometric analyses of the spermatozoa were conducted with a Becton Dickinson FACSCalibur (Becton Dickinson Immunocytometry Systems, San Jose, California) equipped with a 15mW air-cooled argon ion laser and interfaced to a Macintosh Quadra 650 computer (Apple Computer, Inc., Cupertino, California) using BD Cellquest software. Three parameters were used: a green fluorescence (FL1) parameter and two red fluorescence (FL2 and FL3) parameters. Green fluorescence (FL1) was gathered through a 520 nm bandpass filter while red fluorescence parameters were gathered through a 575 nm and a 635 nm bandpass filter, respectively. At least 50.000 spermatozoa were analyzed for each sample.

Dot plots of SYBR®-14/PI-stained spermatozoa showed three populations (Figure 1). The lower right population corresponds to SYBR®-14-stained spermatozoa, the upper right group is double stained (red and green) and the upper left population is PI-stained.

Dot plots of JC-1/SYBR®-14/PI-stained spermatozoa also showed three populations (Figure 2). The upper right population corresponds to membrane-intact spermatozoa with high mitochondrial membrane potential, the upper left group corresponds to membrane-damaged spermatozoa and the lower right population corresponds to membrane-intact spermatozoa with low mitochondrial membrane potential.

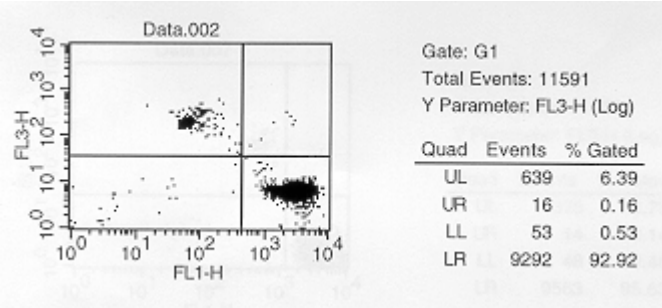

Figure 1. Dot plot of SYBR®-14/PI stained spermatozoa showing three distinct sperm populations: membrane-damaged spermatozoa (red-stained: PI) (upper left), membrane-intact spermatozoa (green-stained: SYBR®-14) (lower right), and moribund spermatozoa stained both red and green (doubly-stained) (upper right).

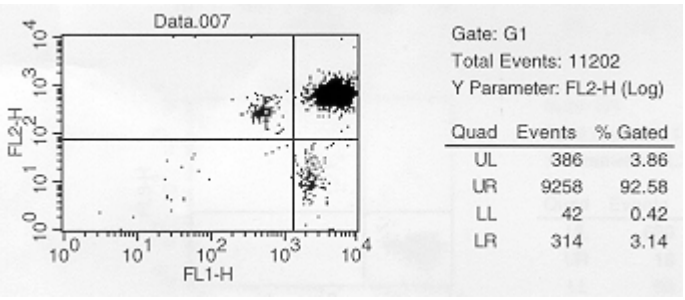

Figure 2. Dot plot of JC-1/SYBR®-14/PI- stained spermatozoa, showing three populations: membrane-intact spermatozoa with high mitochondrial membrane potential (upper right) or low mitochondrial membrane potential (lower right) and membrane-damaged spermatozoa (upper left).

Measurement of sperm DNA fragmentation using the TUNEL assay

DNA fragmentation was detected with a TUNEL assay using the In-Situ Cell Detection Kit from Boehringer Mannheim (Mannheim, Germany).

Spermatozoa were washed twice in 1 ml PVP solution (1 mg/ml polyvinyl-pyrrolidone (PVP) in phosphate-buffered saline (PBS)) by centrifugation at RT (720 x g, 5 min). The final pellet was resuspended in 1 ml PVP solution and 10 µl of 10 x 10^6 spermatozoa/ml was smeared onto a polylysine-coated glass microscope slide and air dried. The slides were washed in PVP solution (5 min) and fixed with 4% paraformaldehyde in PVP solution (pH 7.4) at RT for 30 min. This was followed by permeabilization with a solution of 0.1% Triton X-100 and 0.1% sodium citrate for 2 min on ice. Slides were rinsed twice with PVP solution and air dried. Diluted terminal deoxynucleotidyl transferase (TdT) (25 µl) was mixed with 225 µl nucleotide mixture.

Twenty-five microliter of this TUNEL reaction mixture was added to the spermatozoa on each slide. The samples were incubated in a humidified chamber at 37°C for 60 min in darkness.

Negative (nucleotide solution without TdT), and positive (incubation with 1 mg Dnase I/ml (Pharmacia LKB Biotech) for 10 min at RT before staining with TUNEL reaction mixture) controls were included in all experiments. The slides were rinsed three times with PVP solution and incubated with 25 µl Hoechst 33342 (1µl/ml PVP solution) at RT for 5 min. After washing twice in PVP solution, 100 spermatozoa in different microscopic fields were analyzed by means of fluorescence microscopy. Hoechst stained spermatozoa were first counted (bright blue fluorescence) under ultraviolet light and the percentage of FITC-labelled spermatozoa with fragmented DNA (intense green nuclear fluorescence) was determined by means of Leica DMR epifluorescence microscopy.

Experimental design

Experiment 1: Evaluation of the effect of pH

In the first experiment, the effect of different pH-values of Hepes-TALP on motility, membrane integrity and mitochondrial membrane potential of stored spermatozoa was tested. The pH of Hepes-TALP was measured with a pH-meter (WTW, Weilheim, Germany), which was first calibrated with a pH 7 and a pH 4 standard solution. The initial pH of the medium (pH 7) was adjusted to pH 4, 5 and 6 by the addition of 1 M HCl or to pH 8 by the addition of 1 M NaOH. The amounts of 1M HCl needed to obtain the different pH values were 10, 5 and 3 µl of 1 M HCl/ml medium respectively; pH 8 was reached by the addition of 4 µl of 1 M NaOH/ml medium. The pH-values of the different media were monitored every day. The experiment was repeated three times at 1-week intervals.

Experiment 2: Evaluation of the effect of osmolarity

Different osmolarities of the storage medium were used to test the effect on motility, membrane integrity and morphology of the spermatozoa. Hepes-TALP at pH 6 was adjusted to different osmolarity values. Osmolarity of the solution was evaluated by means of a FISKE ONE-TEN Osmometer (Fiske Associates, Norwood, Massachusetts, USA). The osmolarity of Hepes-TALP (300 mOsm) was adjusted to 400, 500, 600 and 800 mOsm by the addition of sorbitol (Sigma, Bornem, Belgium), or diluted with purified

water to obtain 100 mOsm. The amounts of sorbitol needed to obtain these osmolarities were respectively 0.020, 0.039, 0.056 and 0.093 g/ml medium. Because the addition of sorbitol interfered with flow cytometry, only fluorescence microscopy could be used to analyze the membrane integrity of spermatozoa. Additionally, it was not possible to evaluate the mitochondrial membrane potential by means of flow cytometry.

The osmolarity of each solution was assayed in duplicate. Because the osmolarity has an effect upon sperm morphology, especially tail configuration, sperm morphology was evaluated in this experiment. A smear of spermatozoa was stained with eosin/nigrosin and 100 spermatozoa were evaluated for morphology under a light microscope (Leica DMR) at magnification 1000 x. The experiment was repeated three times at 1-week intervals.

Experiment 3: Evaluation of the effect of sperm storage athmosphere

For this experiment, spermatozoa were prepared as described above and stored in Hepes-TALP at pH 6 and 300 mOsm either under nitrogen-gassed or aerobic conditions. One milliliter of the diluted sperm sample was stored in a 15 ml tube at RT in darkness for 4 days. For storage of spermatozoa under nitrogen-gassed conditions, the tube was filled with dry nitrogen. After 4 days of storage, motility, membrane integrity, mitochondrial membrane potential and DNA integrity of the spermatozoa were investigated. The experiment was repeated three times at 1-week intervals.

Experiment 4: Evaluation of the effect of sperm concentration and of sperm coating during collection

After collection, spermatozoa were separated from seminal plasma by centrifugation (720 x g, 10 min) at RT and the concentration was adjusted with Hepes-TALP to 10×10^6, 100×10^6, 500×10^6 and 1×10^9 spermatozoa/ml or remained undiluted. The concentration was determined by means of a Bürker chamber.

Because sperm survival seems to be severely influenced by sperm concentration, a second series of sperm collection was assessed. Therefore a Triladyl®-diluent (Minitüb, Germany) containing 20% egg yolk and 6.7% glycerol was used to “coat” the sperm membrane during ejaculation in order to protect it from hazardous influences of the seminal plasma. Spermatozoa were collected in a tube containing 5 ml of Triladyl®-diluent (“coated” spermatozoa). Diluent and seminal plasma were separated from the spermatozoa

by centrifugation at 720 x g for 10 min. The same sperm concentrations as above were used.

Statistical Analysis

Differences in membrane integrity, motility, mitochondrial membrane potential, and DNA fragmentation were examined using analysis of variance (ANOVA) methods through Generalized Linear Models (SAS V8, SAS Institute Inc.) for the conditions that were mimicked in the different experimental designs. The agreement between the results obtained by fluorescence microscopic analysis and flow cytometric analysis was investigated with weighted kappa measure of agreement (SAS V8, SAS Institute Inc.) after results were divided into five classes with increasing ranges of 20%.

Results

Experiment 1: Evaluation of the effect of pH

Both fluorescence microscopy and flow cytometry were used for the assessment of membrane integrity of the spermatozoa (Figure 3). The agreement between the results obtained by fluorescence microscopy and by flow cytometry was 87% (SEM = 0.03). This agreement was sufficiently high to limit the assessment of the membrane integrity of spermatozoa to flow cytometry only, which is the preferred method since large numbers of spermatozoa can be analyzed at the same time. As an extra control, 100 spermatozoa were evaluated by means of fluorescence microscopy.

At pH 5, spermatozoa could survive for only 2 days, while at pH 4 the sperm membrane was damaged immediately. After 72 h of incubation, the membrane integrity was best preserved at pH 6 (> 50%) (Figure 3). This percentage was significantly higher than for all other pH - values ($P < 0.05$).

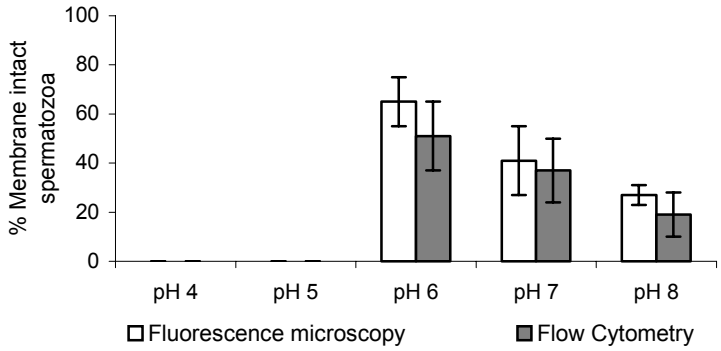

Figure 3. Percentage (mean ± SD, n=3) of membrane-intact spermatozoa after 72 h of incubation in Hepes-TALP at RT at different pH-values as assessed by fluorescence microscopy and flow cytometry

The initial motility of the sperm sample was 85%. Spermatozoa were not able to sustain their motility for more than 72 h at any tested pH - values (Figure 4). During storage, there was no significant difference in motility of spermatozoa incubated in Hepes-TALP either at pH 8, 7 or 6. When stored at pH 4 or 5, spermatozoa immediately became immotile. Because membrane integrity of the spermatozoa was lost immediately, or after 24 h of storage at pH 4 or 5 respectively, spermatozoa stored at these pH-values were therefore classified as non-viable. Interestingly, it was noted that at pH 8, 7 or 6 spermatozoa lost their ability to move over a 72 h storage period, while the percentage of membrane-intact spermatozoa with high mitochondrial membrane potential was lowered more slowly (Figure 4). The pH-value of the media in which the spermatozoa were stored did not change during the experiment indicating that the sperm metabolism did not affect pH (data not shown). Since these experiments showed that incubation of spermatozoa in Hepes-TALP adjusted to pH 6 yielded the best results, we used this medium in the subsequent experiments.

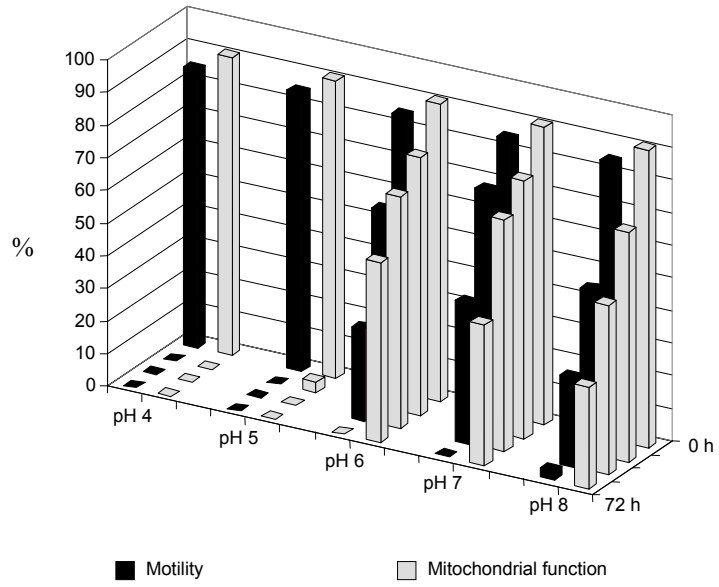

Figure 4. Effect of different pH-values on motility and mitochondrial function of spermatozoa stored in Hepes-TALP for 0, 24, 48 or 72 h (mean, n=3)

Experiment 2: Evaluation of the effect of osmolarity

The effect of different osmolarities of the storage medium on the inhibition of the motility and on the maintenance of the membrane integrity of spermatozoa after 96 h storage is shown in Figure 5. The initial membrane integrity of the sperm samples was 83%. At 300 mOsm 62% of the spermatozoa were membrane-intact: this percentage was significantly higher ($P < 0.05$) than at all other osmolarities. Statistical analysis of the data showed no significant differences between sperm membrane integrity in media varying from 400 to 800 mOsm (between 28 and 36% membrane integrity) after 96 h (Figure 5). The initial motility of the spermatozoa was 85-90%; the total motility of the sperm samples incubated at different osmolarities was less than 15% after 96 h and did not significantly differ among the media except for spermatozoa incubated at 100 mOsm. Normal morphology was well preserved in all sperm samples, except for the hypotonic medium in which membrane-intact spermatozoa showed a curved tail, which was indicative of hypo-osmotic swelling.

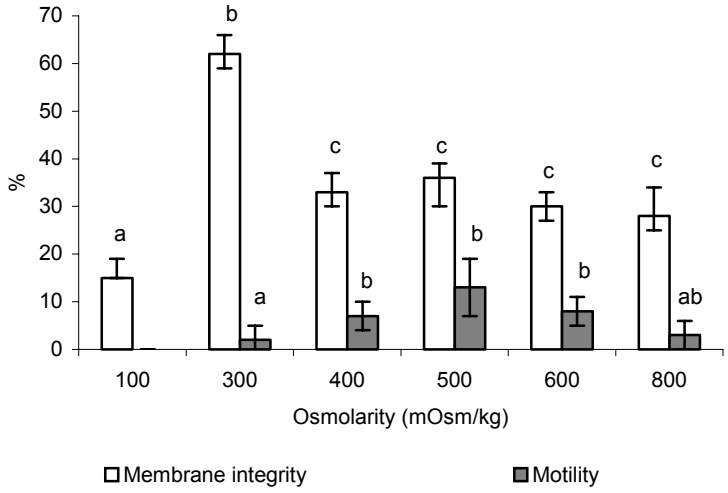

Figure 5. Effect of osmolarity on the percentage of motile and membrane-intact spermatozoa stored for 96 h in Hepes-TALP at pH 6 (Mean ± SD, n=3)

Bars with different superscripts (a,b,c) are significantly different from each other within tested parameter ($P < 0.05$)

Experiment 3: Evaluation of the effect of sperm storage athmosphere

The initial values for the tested parameters were: 85% motility, 87% membrane integrity, 87% high mitochondrial membrane potential and 89% DNA integrity. The results obtained after 96 h are shown in figure 6. No significant differences were observed in motility, membrane integrity, mitochondrial membrane potential and DNA integrity of spermatozoa stored under nitrogen-gassed or aerobic storage condition. Sixty-one percent of the spermatozoa stored under aerobic conditions and 58% of the spermatozoa stored under nitrogen-gassed conditions showed no apoptotic changes in the nuclei after 96 h of storage. Membrane integrity and mitochondrial membrane potential were lost faster than DNA integrity under both conditions.

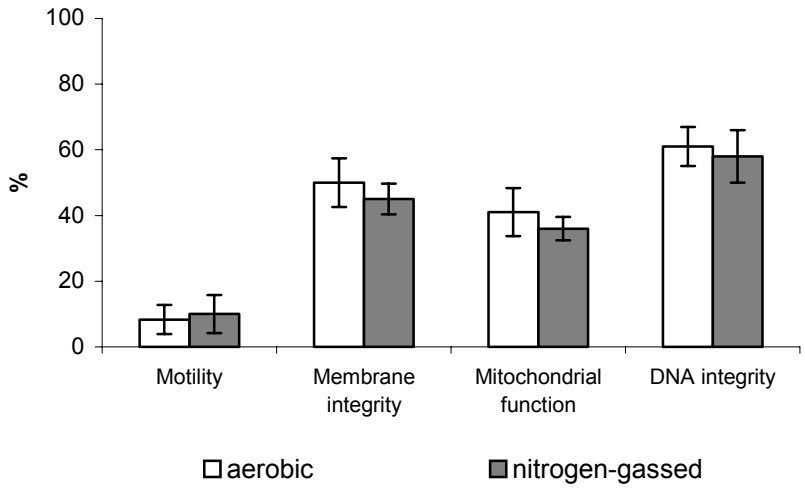

Figure 6. Effect of storage atmosphere on the percentage of motility, membrane integrity, mitochondrial membrane potential and DNA integrity of spermatozoa stored in Hepes-TALP at pH 6 and 300 mOsm for 96 h (Mean ± SD, n=3)

Experiment 4: Evaluation of the effect of sperm concentration and sperm coating during collection

Membrane integrity in samples diluted to 10 x 10^6, 100 x 10^6 or 500 x 10^6 spermatozoa/ml Hepes-TALP was significantly higher than in samples at a concentration of 1 x 10^9 spermatozoa or in undiluted sample ($P < 0.05$) (Figure 7a). However, despite the fact that most spermatozoa were still membrane-intact, sperm motility was greatly decreased after 48 h and almost lost in all sperm samples after 72 h incubation. Motility loss proceeded faster in sperm samples with a concentration exceeding 500 x 10^6 spermatozoa/ml (data not shown). The percentage of spermatozoa with a high mitochondrial membrane potential was low at all sperm concentrations, except for the 10 x 10^6 spermatozoa/ml sample, where most of the membrane-intact spermatozoa maintained a high mitochondrial membrane potentials even after 96 h of incubation (Figure 7a).

In contrast with "uncoated" spermatozoa significantly more "coated" spermatozoa maintained a high mitochondrial membrane potential after 96 h of incubation at concentrations ≥ 100 x 10^6 spermatozoa/ml ($P < 0.05$) (Figure 7b). The number of "coated" spermatozoa with an intact membrane was higher than in the "uncoated" samples, especially in the samples with higher sperm concentrations. In fact, even in the undiluted sample more than 80% of the "coated" spermatozoa remained membrane-intact compared with only 35% for "uncoated" spermatozoa. Unexpectedly, at a sperm concentration of

10 x 10^6, the percentage of spermatozoa with a high mitochondrial membrane potential was significantly higher in "uncoated" (45%) than in "coated" spermatozoa (13%).

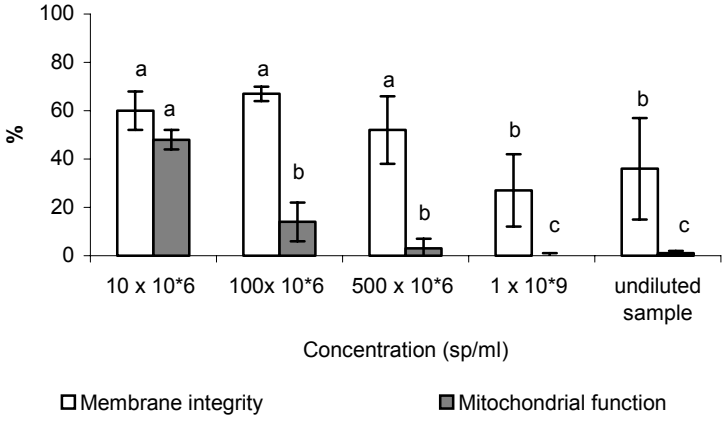

Figure 7a. Effect of sperm concentration on the percentage of membrane-intact spermatozoa and on mitochondrial function of spermatozoa stored in Hepes-TALP at pH 6 and 300 mOsm for 96 h (Mean ± SD, n=3)

Bars with different superscripts (a,b,c) are significantly different from each other within tested parameter ($P < 0.05$)

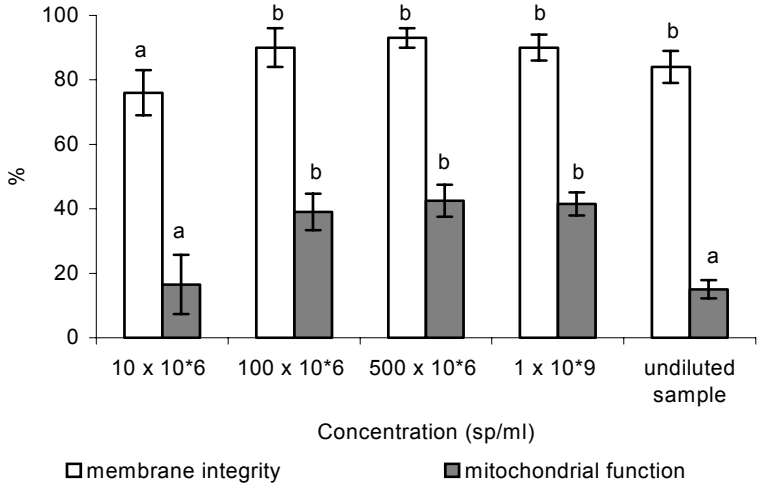

Figure 7b. Effect of sperm concentration and "coating" of the sperm membrane with Triladyl®-diluent on the percentage of membrane-intact spermatozoa and on mitochondrial function of spermatozoa stored in Hepes-TALP at pH 6 and 300 mOsm for 96 h (Mean ± SD, n=3)

Bars with different superscripts (a,b,c) are significantly different from each other within tested parameter ($P < 0.05$)

Discussion

Despite the fact that sperm membrane integrity remains fairly well preserved for several days during liquid storage, motility declines very quickly. Irreversible damage of the motility apparatus and of mitochondrial and nuclear DNA is probably the main reason why semen stored at RT has a declining fertilizing potential. Therefore, we assessed not only sperm membrane integrity and motility, but also investigated sperm mitochondrial membrane potential by means of dye JC-1 and the prevalence of DNA fragmentation in stored spermatozoa as assessed by TUNEL staining. In our study, spermatozoa were stored during 3 or 4 days at RT in a medium based on the zwitterionic organic buffer Hepes.

Sperm membrane integrity and mitochondrial membrane potential were preserved longest when stored in Hepes-TALP at pH 6 for up to 3 days. A decrease in pH of the medium to 6 was well tolerated by spermatozoa, but a lower pH was spermicidal and caused immediate cessation of motility. Interestingly the plasma membrane of the spermatozoa was more able to resist damage caused by pH-changes than the mechanisms responsible for sperm motility. A possible explanation could be that the intracellular pH (pH_i) of the spermatozoa decreased to the extracellular pH (pH_e), which is the case in human, ram and boar semen (Gatti et al., 1993; Olmstedt et al., 2000). This suggests that spermatozoa have minimal ability to maintain their pH in acidic environment and that their membrane is permeable to protons. There is evidence that pH_i regulates sperm motility in invertebrate species (Nishioka and Cross, 1978) although no important regulatory mechanisms for controlling pH_i, such as Na^+/H^+ or K^+/H^+ exchange are present in mammalian spermatozoa (Hamamah and Gatti, 1998; Jones and Bavister, 2000). Carr et al. (1985) suggested that the low pH of the cauda epididymal plasma in combination with the presence of a permeable weak acid like lactic acid are responsible for the short-term quiescence of the epididymal spermatozoa by depressing their pH_i. However, when ejaculated spermatozoa are stored for longer than 48 h, reactivation of sperm motility is much more difficult after immobilization by low pH (Jones and Bavister, 2000).

The second tested parameter in this study was the osmolarity of the medium. Sorbitol was used to increase the osmolarity of the medium: this osmolyte may cross the plasma membrane to a limited extent and is an appropriate metabolic substrate for spermatozoa (Clegg et al., 1986; Crichton et al., 1994). Although caudal epididymal plasma of most animals is hyperosmotic to blood (300 mOsm) with an average of 354 mOsm (Verberckmoes et al., 2001), increasing the osmotic pressure had no positive

influence on membrane integrity of bull spermatozoa and actually even caused a significant drop in percentage surviving spermatozoa compared to isotonic values. Hyperosmotic conditions are present in epididymal plasma of hibernating bats (Crichton et al., 1994) and it has been hypothesized that dehydration of bat spermatozoa would reduce their metabolic needs and thereby prolong their survival. This could not be replicated with bovine spermatozoa because dehydration of the spermatozoa in hyperosmotic solutions caused membrane damage. Hypo-osmotic solutions (< 300 mOsm) were even more deleterious to bull sperm motility because the influx of water causes the mitochondria in the tail to swell, an increase in volume and bursting (Drevius and Ericksson, 1966). This explains the decrease in membrane integrity and motility of the spermatozoa stored in Hepes-TALP of 100 mOsm.

Although spermatozoa are stored under low oxygen tension in the cauda epididymidis, no significant differences were observed in motility, mitochondrial membrane potential and membrane integrity of spermatozoa stored under nitrogen-gassed conditions compared to aerobic conditions after 4 days. In an analogous study performed by Krzyzosiak et al. (2001), membrane integrity of spermatozoa was better preserved when stored in CAPROGEN® (Livestock Improvement Corporation, Hamilton, New Zealand) under nitrogen-gassed conditions, however no significant differences were observed in the motility and fertility of the spermatozoa. Our study showed that ageing of spermatozoa in vitro leads to a greater incidence of injury in mitochondria than in nuclear DNA. This confirms that changes in mitochondrial membrane potential occur during the early stages of apoptosis and could be detectable before DNA fragmentation occurs (Donnelly et al., 2000). The decline could also be explained by the inability of mtDNA to auto-repair and by the fact that the rate of mitochondrial ageing is faster than that of nuclear DNA (Cummins et al., 1994). In contrast to expectations, no differences were observed in DNA damage of spermatozoa stored for 4 days under either aerobic or nitrogen-gassed conditions. Since the generation of reactive oxygen species (ROS) and consequently DNA damage is likely to be higher under aerobic conditions, it was surprising to find no significant differences in mitochondrial and DNA fragmentation in spermatozoa stored under either aerobic or nitrogen-gassed conditions. This may indicate that either ROS formation was not reduced under nitrogen-gassed conditions, or that its effect on apoptosis is minimal. Both hypotheses need further investigation. Because storage under nitrogen-gassed conditions caused no important improvement in sperm quality parameters, aerobic storage conditions were used in the last experiment.

Sperm concentration also greatly influences sperm survival. In the epididymis, spermatozoa are stored at a concentration of 1 x 10^9 to 3 x 10^9 spermatozoa/ml. Ejaculated and stored spermatozoa on the contrary, preserve their membrane integrity best at lower sperm concentrations. The reason may be related to the reduced output of toxic metabolic products or to a slower exhaustion of substrate at sperm high dilutions. In vitro, substrates become depleted rapidly and toxic waste products accumulate, whereas in vivo inputs are limited to easily metabolizable substrates from the external environment and toxic products are disposed off systematically (Van Demark and Couturier, 1958). The most important toxic products accumulated during in vitro storage are ROS formed by the oxidative phosphorylation system of the inner mitochondrial membrane (Miquel and Fleming, 1986). In our study, it is evidenced that storage of spermatozoa at high concentrations was less detrimental at all when spermatozoa were "coated" with Triladyl®- diluent before being stored. Collection of spermatozoa in Triladyl®- diluent during ejaculation has been the method of choice to limit the contact of spermatozoa with seminal plasma. Bovine seminal plasma is notorious for its negative influence on sperm survival in vitro (Shannon and Curson, 1983, 1987), because it contains a group of acidic proteins (Bovine seminal plasma (BSP) proteins) that bind to the spermatozoa (Desnoyers and Manjunath, 1992). Their interaction with spermatozoa leads to the efflux of choline phospholipids and cholesterol from the sperm plasma membrane, which appears to be an important step in the capacitation process (Therien et al., 1998; Moreau et al., 1999). Since cholesterol is recognized to have a stabilizing effect on membranes (Yeagle, 1985), this efflux would be expected to cause destabilization of the membrane. Other cationic peptides present in seminal plasma are also known to bind to spermatozoa (which has a net negative charge) (Yanagimachi et al., 1972) and to destabilize the plasma membrane. The water-soluble cationic fraction of egg yolk protects against the detrimental effect of seminal plasma components (Shannon and Curson, 1972,1983) by competing in binding to the sperm membrane. The low-density lipoprotein fraction of egg yolk has also been shown to reduce the loss of acrosomal enzymes and to prevent degenerative changes in the acrosome during liquid storage (Salamon and Maxwell, 2000).

The very simple action of "coating" spermatozoa for less than 5 min with egg yolk containing diluent during collection had an important effect upon several characteristics after 4 days of storage in Hepes-TALP at pH 6 and 300 mOsm. These results are very promising for the improvement of preservation and fertilizing ability of liquid bovine semen in the future. Consequently, the effect of sperm "coating" during ejaculation on the

fertilizing ability of spermatozoa stored in vitro during several days, is actually under investigation in our laboratory.

Acknowledgments

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5.

EFFECT OF SPERM COATING ON THE SURVIVAL AND PENETRATING ABILITY OF IN VITRO STORED BOVINE SPERMATOZOA

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Abstract

The aim of this study was to examine the effect of sperm "coating" on the survival and penetrating ability of in vitro stored bovine spermatozoa. Semen was collected by means of an artificial vagina connected with a tube containing 5 ml of the commercial Triladyl®-diluent. Both diluent and seminal plasma were removed by centrifugation (720 x g, 10 min) and spermatozoa were stored under different in vitro storage conditions. In the first and second experiment, "control" and "coated" spermatozoa were stored in Hepes-TALP (pH 6 and 7) at room temperature (RT). After 4 days of storage, the progressive motility, membrane integrity, mitochondrial membrane potential and DNA integrity of the spermatozoa were evaluated before and after Percoll®-centrifugation. The in vitro oocyte penetration rate of spermatozoa was examined only after Percoll®-centrifugation. A significantly ($P < 0.05$) positive effect of sperm "coating" was observed on the tested sperm characteristics and oocyte penetration rate of spermatozoa when they were stored in Hepes-TALP at pH 7, but not at pH 6. In the last experiment, the effect of storage medium: Hepes-TALP (pH 7) or Triladyl®-diluent was investigated on motility, membrane integrity, mitochondrial membrane potential and in vitro oocyte penetration potential of "coated" spermatozoa stored at RT or at 4°C for up to 4, 5 and 6 days. After 6 days of storage in Triladyl®, a significantly ($P < 0.05$) higher percentage of motile and membrane intact spermatozoa with high mitochondrial membrane potential was obtained at both temperatures leading to a significantly higher in vitro oocyte penetration rate. These results indicate that sperm "coating" could preserve sperm characteristics and oocyte penetrating capacity of bovine spermatozoa stored in egg yolk containing diluent for up to 6 days.

Introduction

Liquid semen can be used as an alternative for artificial insemination with frozen-thawed spermatozoa, on the condition that its fertilizing potential can be maintained for a minimum of 2 days and preferably 4 days to provide for easy transport and use in distant locations (Vishwanath and Shannon, 2000). Much research has already been done to prolong the in vitro viability and fertilizing potential of stored liquid semen by altering or reducing the catabolic metabolism of spermatozoa or by increasing the stability of sperm membranes to thermal and other environmental insults (Foote and Parks, 1993). However, until now no important improvements have been made to increase the fertilizing potential of spermatozoa longer than 3 to 5 days when they were stored in liquid environment at high dilutions at ambient temperatures (Vishwanath and Shannon, 1997).

A novel method to minimize the damage of ejaculated spermatozoa was obtained by “coating” spermatozoa for less than 5 minutes during collection with the commercial Triladyl®-diluent (De Pauw et al., 2003). With this method, the contact between spermatozoa and seminal plasma was limited by collecting spermatozoa in a tube containing 5 ml of Triladyl®-diluent, which was connected to an artificial vagina. The water-soluble cationic fraction of egg yolk is able to protect both sperm plasma and acrosome membrane against the detrimental effect of seminal plasma components (Shannon and Curson, 1972, 1983). This competing in binding with seminal plasma components prevents the sperm plasma membrane against the efflux of choline phospholipids and cholesterol, which causes destabilization of the membrane. Furthermore, the protective action of low-density lipoprotein binding could also be responsible for the stabilization of the plasma membrane through the critical zones (Vishwanath et al., 1992) and for the improvement of motility after storage (Moussa et al., 2002).

Furthermore, it would also be valuable to assess deterioration due to changes caused by ageing of stored spermatozoa (Maxwell and Salamon, 1993; Paulenz et al., 2002) resulting in sperm plasma membrane disruption, loss of mitochondrial membrane potential and DNA fragmentation. These are all hallmarks of apoptosis (Donnelly et al., 2000) leading to a progressive loss of fertilizing ability over storage time (Cumming et al., 1994).

The aims of the study were 1) to analyze the effect of sperm “coating” during collection on the progressive motility, membrane integrity, mitochondrial membrane potential and DNA integrity of spermatozoa stored in Hepes-TALP at pH 6 or 7 at RT for

4 days, 2) to examine the in vitro oocyte penetration rate of 4 days old "control" and "coated" spermatozoa stored at RT, 3) to evaluate the effect of the storage medium (Hepes-TALP (pH 7) and Triladyl®-diluent) on the oocyte penetration rate of "coated" spermatozoa stored at 4°C and at RT for up to 4, 5 and 6 days.

Materials and Methods

Media

Chemicals and media were obtained from Sigma (Bornem, Belgium) and GIBCO™ Invitrogen Corporation (Merelbeke, Belgium).

Hepes-TALP containing 114 mM NaCl, 3.1 mM KCl, 0.3 mM NaH_2PO_4, 2.1 mM $CaCl_2$, 0.4 mM $MgCl_2$, 2 mM $NaHCO_3$, 0.2 mM sodium pyruvate, 10 mM sodium lactate, 10 µg/ml gentamycin sulphate, 10 mM Hepes and 3 mg/ml Bovine Serum Albumin.

Triladyl®-diluent (Minitüb, Germany), a TRIS-based diluent supplemented with 20% egg yolk and 6.7 % of glycerol. Final concentrations of antibiotics per 100 ml were: 5 mg tylosin, 25 mg gentamycin, 30 mg spectinomycin and 15 mg lincomycin.

Maturation medium consisting of modified bicarbonate buffered TCM199 medium supplemented with 20% heat-inactivated foetal calf serum (N.V. HyClone Europe S.A., Erembodegem, Belgium), 0.2 mM sodium pyruvate, 50 µg/ml gentamycin sulphate and 0.4 mM glutamin.

Fertilization medium containing Tyrode's solution without Hepes supplemented with 25 mM $NaHCO_3$, 0.2 mM sodium pyruvate, 10 mM sodium lactate, 10 µg/ml gentamycin sulphate, 6 mg/ml fatty acid-free BSA and 10 µg/ml heparin.

Collection and preparation of semen

Spermatozoa from a 3-year-old Red Pied bull were collected by means of an artificial vagina connected with an empty tube ("control" spermatozoa). Within 5 minutes, a second ejaculate was collected following the same method but in a tube containing 5 ml Triladyl®-diluent ("coated" spermatozoa). Immediately after collection, both ejaculates were centrifuged (720 x g, 10 min) at RT to remove the supernatant. The sperm

concentration of the pellet was determined using a Bürker chamber and subsequently diluted to a concentration of 10 x 10^6 spermatozoa/ml in Hepes-TALP (pH 6 or 7) or in Triladyl®-diluent according to the experiment. Spermatozoa were stored both at RT and 4°C in darkness until fertilization. They were either analyzed directly (Experiment 1) or after Percoll®-centrifugation (Experiment 2 and 3). Motile and membrane-intact spermatozoa were separated by centrifugation (720 x g, 30 min) at RT on a discontinuous Percoll®-gradient (Pharmacia, Uppsala, Sweden) composed of 2 ml each of 90% and 45% Percoll solutions in Hepes buffered Sp-TALP without BSA. After removal of Percoll® supernatant, the sperm pellet was diluted in 5 ml Hepes buffered Sp-TALP supplemented with BSA and centrifuged (180 x g, 10 min).

Processing and analysis of sperm samples

Motility

The percentage of total and progressive motile spermatozoa was subjectively assessed by visual estimation of two investigators. Eight microliter of the sperm sample was placed on a prewarmed glass slide, covered with a glass cover slip, and viewed under a Leica DMR light microscope (Van Hopplynus NV, Brussels, Belgium) (magnification 200 x) equipped with a stage warmer (37°C).

Membrane integrity

The nucleic stains SYBR®-14 and propidium iodide (PI) were used for analyzing membrane integrity of the spermatozoa (LIVE/DEAD® Sperm Viability Kit (Molecular Probes)) and evaluated by means of fluorescence microscopy (magnification 1000 x) at 37°C. SYBR®-14 labeled DNA of membrane-intact spermatozoa, resulting in a bright green fluorescence when excited at 488 nm, while PI only stained the nuclei of membrane-damaged spermatozoa red (excitation: 530 nm). The membrane integrity of at least 100 spermatozoa was evaluated.

Mitochondrial membrane potential

For evaluation of mitochondrial membrane potential, spermatozoa were labeled with JC-1 (Molecular Probes, Leiden, The Netherlands). This fluorophore exhibits

potential-dependent accumulation in mitochondria, and can reversibly change its emission from green to orange with increasing transmembrane electrical potential. This staining was combined with SYBR®-14 and PI. Two microliters of 1.53 mM JC-1 (Molecular Probes) in DMSO was added to 1 ml of the diluted sperm sample in combination with 0.7 µl SYBR®-14 and 12 µl of 2.99 mM PI (Thomas et al., 1998; Garner and Thomas, 1999).

DNA fragmentation

Measurement of sperm DNA fragmentation was performed using the TUNEL assay (In-situ Cell Detection Kit, Boehringer Mannheim, Mannheim, Germany).

Spermatozoa were washed twice in PVP solution (1 mg/ml polyvinyl-pyrrolidone (PVP) in phosphate-buffered saline)) by centrifugation at RT (720 x g, 5 min). The final pellet was resuspended in PVP solution and 10 µl of 10 x 10^6 sp/ml was smeared onto a polylysine-coated glass microscope slide and air dried. The slides were washed in PVP solution (5 min) and fixed with 4% paraformaldehyde in PVP solution (pH 7.4) at RT for 30 min. This was followed by permeabilization with a solution of 0.1% Triton X-100 and 0.1% sodium citrate for 2 min on ice. Slides were rinsed twice with PVP solution and air dried. Twenty-five microliters diluted terminal deoxynucleotidyl transferase (TdT) was mixed with 225 µl nucleotide mixture. Twenty-five µl of this TUNEL reaction mixture was added to the spermatozoa on each slide. The samples were incubated in a humidified chamber at 37°C for 60 min in darkness.

Negative (nucleotide solution without TdT), and positive (incubation with 1 mg Dnase I/ml (Pharmacia LKB Biotech) for 10 min at RT before staining with TUNEL reaction mixture) controls were included in all experiments. The slides were rinsed three times with PVP solution and incubated with 25 µl Hoechst 33342 (1µl/ml PVP solution) at RT for 5 min. After washing twice in PVP solution, 100 spermatozoa in different microscopic fields were analyzed by means of fluorescence microscopy. Hoechst stained spermatozoa were first counted (bright blue fluorescence) under ultraviolet light and the percentage of FITC-labelled cells with fragmented DNA (intense green nuclear fluorescence) was determined by means of Leica DMR epifluorescence microscopy (magnification 1000 x).

In vitro oocyte maturation and fertilization

Cow ovaries were randomly collected from a local abattoir and transported to the laboratory within 2 h. Follicles of 2 to 6 mm in diameter were aspirated and cumulus-oocyte complexes (COC) were matured in 500 µl maturation medium at 38.5 °C in 5% CO_2 in air at 100% humidity for 18-26 h (Van Soom et al., 2002). After maturation, 50 oocytes were incubated with a final sperm concentration of 10^6 spermatozoa/ml for 20 h.

For the assessment of oocyte penetration rate, the presumed zygotes were vortexed during 2 min in 2 ml Hepes-TALP, to remove excess spermatozoa and cumulus cells, and fixed in 2% glutaraldehyde and 2% paraformaldehyde in phosphate buffered saline (PBS) for at least 24 h. After fixing, zygotes were stained with 10 µg/ml Hoechst 33342 (Molecular Probes, Leiden, The Netherlands) during 10 min and evaluated with a Leica DMR fluorescence microscope for signs of penetration. Successful oocyte penetration was characterized by the presence of maternal and paternal pronuclei. Oocytes with more than two pronuclei were also considered penetrated (Ohgoda et al., 1988).

Experimental design

Experiment 1: Difference in sperm characteristics of “control” and “coated” spermatozoa stored in Hepes-TALP at pH 6 or 7 at RT for up to 4 days

“Control” and “coated” spermatozoa were collected and prepared as mentioned above. After 4 days of storage in Hepes-TALP at pH 6 or 7 at RT, the progressive motility, membrane integrity, mitochondrial membrane potential and DNA integrity of the stored spermatozoa were evaluated. This experiment was repeated three times at 1-week intervals.

Experiment 2: Difference in sperm characteristics and oocyte penetrating ability of “control” and “coated” spermatozoa stored in Hepes-TALP at pH 6 or 7 at RT for up to 4 days

For this experiment, “control” and “coated” spermatozoa were stored at a concentration of 10 x 10^6 spermatozoa/ml Hepes-TALP (pH 6 or 7) at RT. For each sperm sample, 20 ml was stored with final sperm concentration of 200 x 10^6 spermatozoa. After 4 days of storage, sperm samples were centrifuged (720 x g, 10 min) at RT and the supernatant was removed. Motile and membrane-intact spermatozoa in the pellet were

separated on a discontinuous Percoll®-gradient. After washing, the sperm pellet of all samples was evaluated for membrane integrity, progressive motility and mitochondrial membrane potential as mentioned above. The DNA integrity of the sperm samples was evaluated on the original sample and not after Percoll®-centrifugation because this sperm preparation method eliminates dead and dying cells and isolates spermatozoa with best DNA integrity (Donnelly et al., 2000), and because of the high number of spermatozoa needed for evaluation. The number of spermatozoa present in the pellet (% sperm concentration) was counted with a Bürker chamber and expressed in relation to the total number of spermatozoa (= 200 x 10^6) used for Percoll centrifugation. When the sperm concentration in the pellet was lower than 10%, also spermatozoa recovered at the interface of the 45 and 90% Percoll layers were used for in vitro oocyte penetration experiments. The sperm concentration was adjusted to obtain a final sperm concentration of 10^6 spermatozoa/ml fertilization medium. Cumulus oocyte complexes (COC) (n = 772, 3 replicates) were co-incubated with spermatozoa of different treatment groups for 20 h. Finally, the presumed zygotes were vortexed, fixed, stained with Hoechst 33342 and examined with a Leica DMR fluorescence microscope for signs of oocyte penetration. This experiment was repeated three times at 1-week intervals.

Experiment 3: Difference in oocyte penetrating ability of spermatozoa stored in Hepes-TALP (pH 7) or Triladyl®-diluent at RT or at 4°C for up to 4, 5 and 6 days

Immediately after collection, “control” and “coated” spermatozoa were evaluated and stored at a concentration of 10 x 10^6 spermatozoa/ml. For each sperm sample, 200 x 10^6 spermatozoa were stored in 20 ml Hepes-TALP (pH 7) or in 20 ml Triladyl®-diluent. Storage was performed at RT and at 4°C in darkness. After storage for 4, 5 and 6 days, spermatozoa were separated on a discontinuous Percoll®-gradient. The progressive motility, membrane integrity and mitochondrial membrane potential of spermatozoa in the pellet were evaluated. Spermatozoa recovered from the interface of the Percoll®-gradient were only used when spermatozoa were stored in Hepes-TALP for up to 6 days. For each sperm storage condition, 50 matured COC were incubated with 10^6 spermatozoa/ml fertilization medium for 20 h. Finally, the presumed zygotes were vortexed, fixed, stained with Hoechst 33342 and examined with a Leica DMR fluorescence microscope for signs of oocyte penetration. This experiment was repeated three times at 1-week intervals.

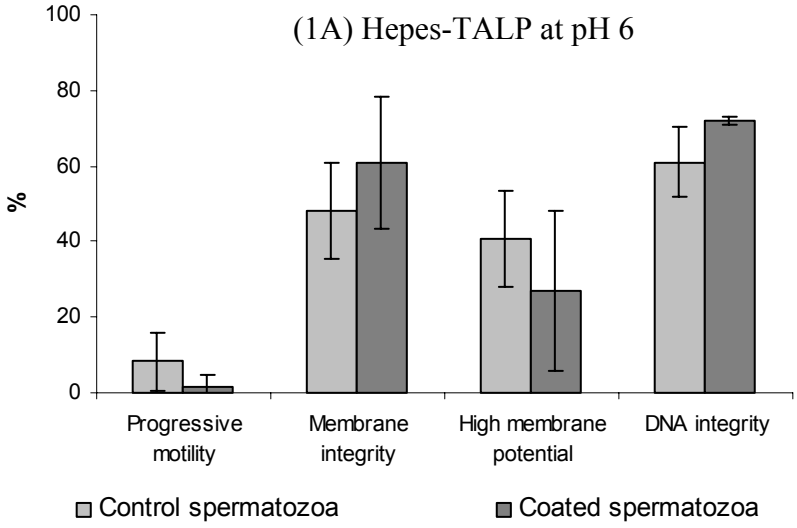
Statistical analysis

Differences in membrane integrity, motility, mitochondrial membrane potential, DNA integrity or oocyte penetrating ability of “control” and “coated” spermatozoa were examined using analysis of variance (ANOVA) methods through Generalized Linear Models. Statistical analysis was performed with procedures available in SPSS 10.0 (Chicago, USA). Values were considered to be statistically significant when $P < 0.05$.

Results

Experiment 1: Difference in sperm characteristics of “control” and “coated” spermatozoa stored in Hepes-TALP at pH 6 or 7 at RT for up to 4 days

The initial percentage of progressively motile spermatozoa was 85%. After 4 days, no significant difference in progressive motility was observed between “control” and “coated” sperm samples when stored at pH 6 (Figure 1). A significantly higher percentage of membrane- and DNA-intact spermatozoa, and spermatozoa with high mitochondrial membrane potential was observed in “coated” than in “control” sperm samples when stored at pH 7 ($P < 0.05$, Figure 1B). However, no significant effect of collection method on these sperm characteristics was observed when spermatozoa were stored in Hepes-TALP at pH 6 (Figure 1A).



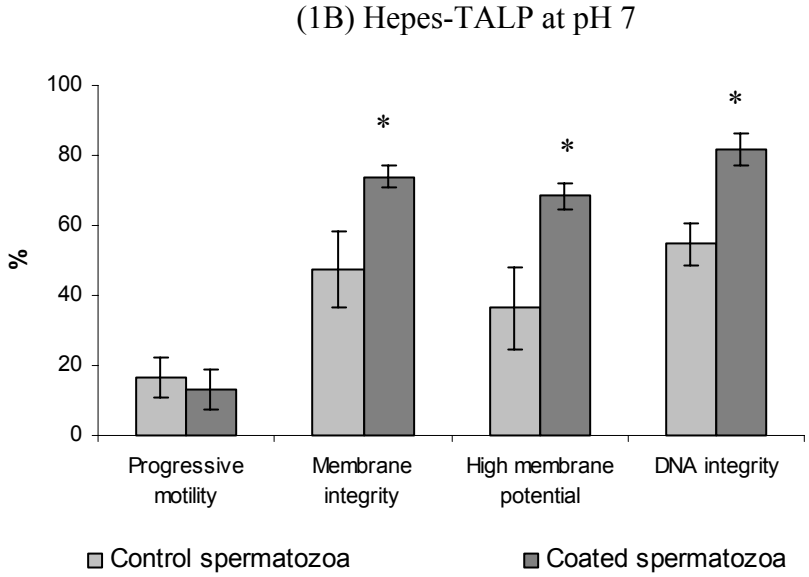


Figure 1. Progressive motility, membrane integrity, mitochondrial membrane potential and DNA integrity of "control" and "coated" spermatozoa stored at RT in (A) Hepes-TALP at pH 6 or (B) Hepes-TALP at pH 7 for up to 4 days. (Mean of 3 replicates ± SEM)

*Value of the tested sperm characteristic is significantly different between "control" and "coated" spermatozoa ($P < 0.05$)

Experiment 2: Difference in sperm characteristics and oocyte penetrating ability of "control" and "coated" spermatozoa stored in Hepes-TALP at pH 6 or at 7 at RT for up to 4 days

The percentage of motile and membrane-intact spermatozoa with high mitochondrial membrane potential present in the pellet after discontinuous Percoll® gradient centrifugation is shown in Figure 2. After Percoll centrifugation, "coated" spermatozoa stored at pH 7 showed a significantly higher percentage of progressive motility (83%), of membrane integrity (91%) and of high mitochondrial membrane potential (91%) (Figure 2B, $P < 0.05$) than "control" spermatozoa (progressive motility: 43%; membrane integrity and high mitochondrial membrane potential: 70%). Furthermore, significantly ($P < 0.05$) more "coated" spermatozoa were recovered after Percoll®-gradient separation.

However, "coating" of spermatozoa was not effective to preserve sperm quality when storage was performed at pH 6. None of the tested sperm characteristics were

significantly different between “control” and “coated” spermatozoa when stored for up to 4 days (Figure 2A).

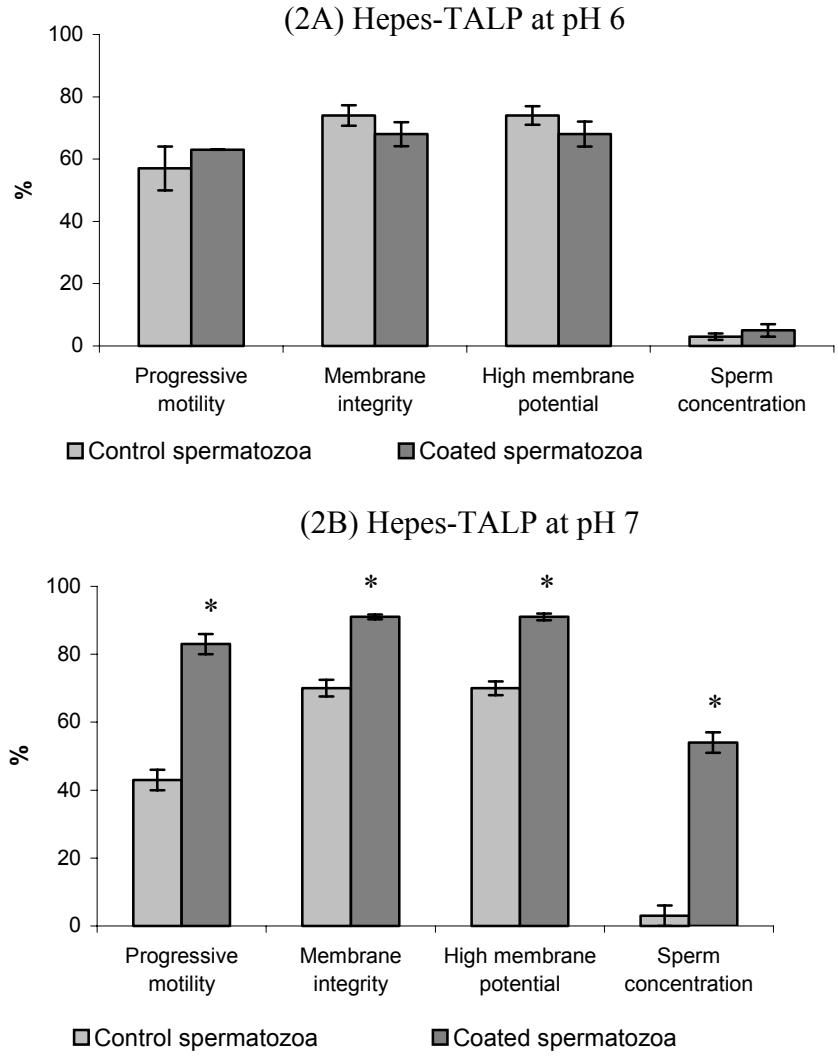


Figure 2. Progressive motility, membrane integrity, high mitochondrial membrane potential and sperm concentration present in the pellet after Percoll®-centrifugation of “control” and “coated” spermatozoa at RT in (A) Hepes-TALP at pH 6 or (B) Hepes-TALP at pH 7 stored for up to 4 days. (Mean of 3 replicates ± SEM)

*Value of the tested sperm characteristic is significantly different between “control” and “coated” spermatozoa ($P < 0.05$)

“Coated”spermatozoa stored at pH 7 displayed a significantly (Figure 3, $P < 0.05$) higher potential to penetrate oocytes (77%) than “control” spermatozoa (20%). In fact they penetrated oocytes much better than frozen-thawed spermatozoa (56%; data not shown). Unexpectedly, the beneficial effect of “coating” was reversed when spermatozoa were stored at pH 6.

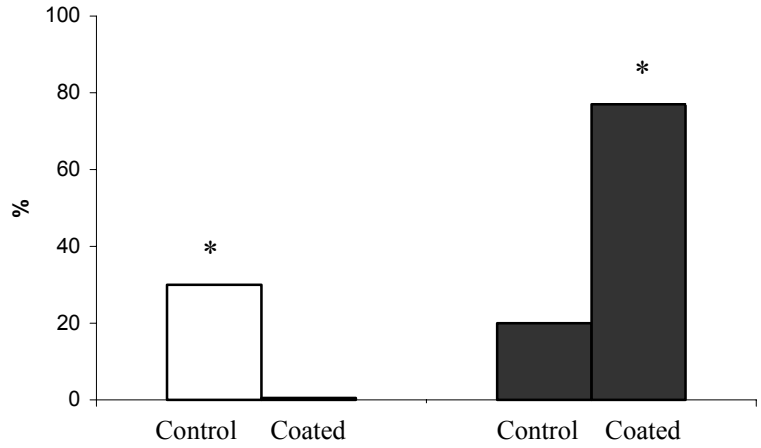


Figure 3. Oocyte penetration rate of "control" and "coated" spermatozoa stored in Hepes-TALP at pH 6 (□) or pH 7 (■) for up to 4 days and after Percoll®-centrifugation (Mean of 3 replicates).

*Value of oocyte penetration rate is significantly different between "control" and "coated" spermatozoa ($P < 0.05$).

Experiment 3: Difference in oocyte penetrating ability of spermatozoa stored in Hepes-TALP (pH 7) or Triladyl®-diluent at RT or at 4°C for up to 4, 5 and 6 days

The initial percentage of total and progressively motile spermatozoa was 80%. This percentage remained similar for up to 6 days when "coated" spermatozoa were stored in Triladyl®-diluent, independent of the temperature (Figure 4A and B). A different evolution was observed when sperm storage took place in Hepes-TALP. At RT, both total and progressive motility decreased to reach a value of 40% at day 6, while at 4°C the progressive and total motility of the sperm sample declined more rapidly and were completely lost after 4 and 6 days of storage respectively (Figure 4A and B).

The initial percentage of membrane-intact spermatozoa with high mitochondrial membrane potential was 87%. As shown in Figure 5 (A and B), no decrease was observed after 6 days when spermatozoa were stored in Triladyl®-diluent at both temperatures. After 6 days, the percentage of membrane-intact spermatozoa with high mitochondrial membrane potential was significantly ($P < 0.05$) lower when spermatozoa were stored in Hepes-TALP at RT (62 %) and 4°C (72%) in comparison with storage in Triladyl®-diluent at the same temperature.

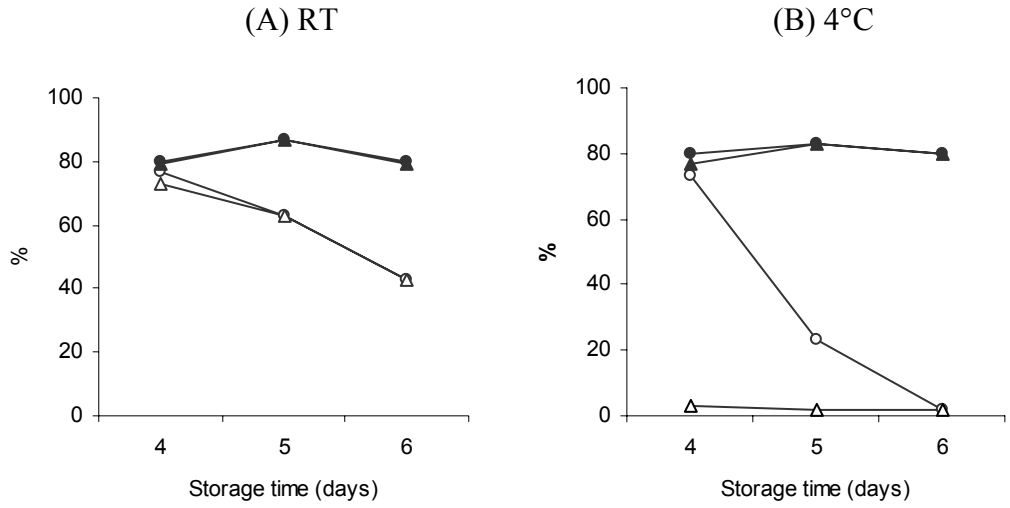


Figure 4. Effect of storage medium Hepes-TALP (white) and Triladyl®-diluent (black) on the percentage total (○ and ●) and progressive (6 and ▲) motile spermatozoa stored at (A) RT or (B) 4°C for up to 4, 5 or 6 days

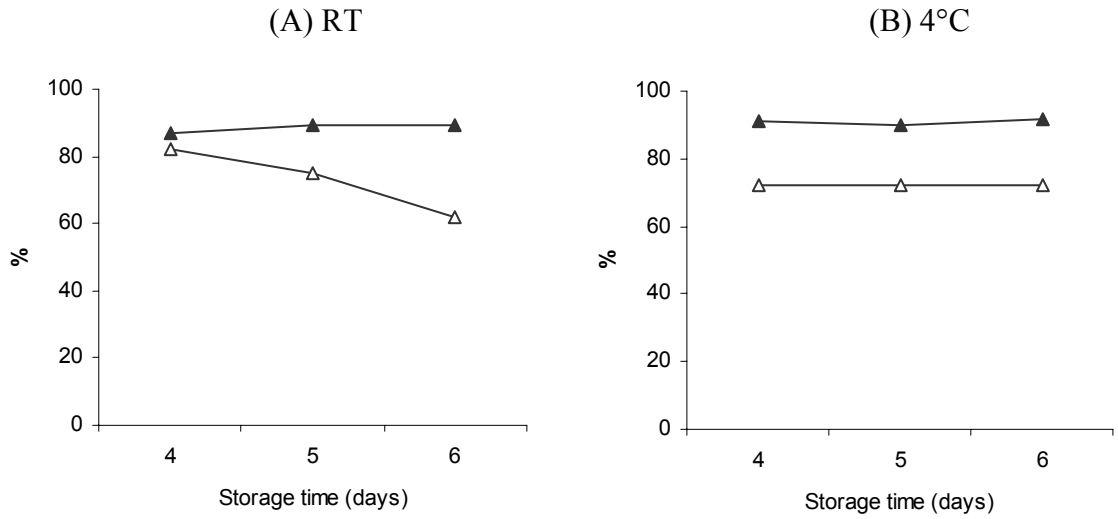


Figure 5. Effect of storage medium Hepes-TALP (6) and Triladyl®-diluent (4) on the percentage membrane-intact spermatozoa with high mitochondrial membrane potential stored at (A) RT or (B) 4°C for up to 4, 5 or 6 days

No significant difference was observed in oocyte penetration rate of spermatozoa stored in the two media for up to 5 days at 4°C and at RT (Figure 6). A significant difference was observed on day 6 at both temperatures. The percentage of oocytes penetrated by spermatozoa stored in Triladyl®-diluent (89% at RT; 71% at 4°C) for up to 6 days was significantly higher ($P < 0.05$) than after storage in Hepes-TALP at pH 7 (53% at RT, 3% at 4°C).

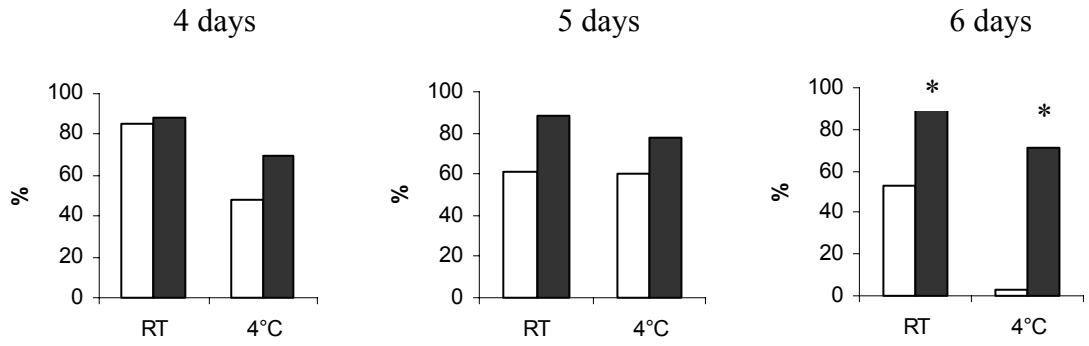


Figure 6. Effect of storage medium: Hepes-TALP (□) or Triladyl®-diluent (■) on oocyte penetration rate of spermatozoa stored at RT or 4°C for up to 4, 5 or 6 days (Mean of 3 replicates).

*Oocyte penetration rate is significantly different between the media and within the same temperature ($P < 0.05$).

Discussion

From the results of the present study, it is clear that a minimal change in sperm collection method can have a profound influence on sperm survival in vitro. Spermatozoa are easily damaged after ejaculation, mainly due to changes caused by exposure to seminal plasma or by handling of semen during the lowering of temperature (Graham, 1994; Paulenz et al., 2002). This damage can lead to detrimental effects on motility parameters and membrane integrity of spermatozoa followed by an inevitable reduction in their fertilizing potential. These effects may be minimized using appropriate dilution and collection medium (Maxwell and Johnson, 1999). However, irrespective of the diluent, dilution rate, temperature or storage conditions, spermatozoa deteriorate as the duration of storage increases (Maxwell and Salamon, 1993). This leads to irreparable damage to mitochondria or to haploid genome in the nucleus, resulting in a non-viable conceptus (Vishwanath and Shannon, 1997). The number of apoptotic spermatozoa in fresh ejaculates varies among different bulls and makes up less than 17% of the total number of spermatozoa (Anzar et al., 2002). This number increases during storage, therefore not only the progressive motility and membrane integrity of the sperm samples was evaluated, but special attention was also paid to ageing-related functional changes within the mitochondria and nuclear DNA of stored spermatozoa. Because no internal repair mechanism exists in spermatozoa, an extraneous supply of protectants, or an environment where damage is minimized, is essential to maintain its fertilizing potential (Vishwanath and Shannon, 1997).

In our experiments, an attempt was made to prolong the survival of stored spermatozoa by stabilizing the sperm plasma membrane and by protecting them against the destructive activity of seminal plasma. Bovine seminal plasma can cause permanent changes in ejaculated spermatozoa (Graham, 1994), even after exposure times of less than 15 min (Dott et al., 1979). Prolonged exposure markedly reduces sperm quality and permanently diminishes their fertilizing capacity (Mortimer, 1994). Therefore, it is essential that seminal plasma is removed quickly and efficiently. Washing spermatozoa by centrifugal sedimentation and resuspension in fresh medium is generally held to be the quickest and most effective method of removing seminal plasma, however this method can cause damage to the spermatozoa (Harrison and White, 1972). In our experiments, the contact time between seminal plasma and spermatozoa was limited by collecting and diluting bull semen directly in Triladyl®-diluent supplemented with egg yolk and glycerol. The protective mechanism of egg yolk against the detrimental components of seminal plasma seems to rely on the low-density lipoproteins, which protect spermatozoa (Shannon and Curson, 1983) by competing in binding to the sperm membrane. This binding of egg yolk lipoproteins is rather effective, since its outcome was still visible after 4 days of storage in a simple defined salt solution Hepes-TALP (pH 7). This was evidenced by the fact that approximately 30% more spermatozoa of the “coated” sperm sample maintained an intact membrane, a high mitochondrial membrane potential and intact DNA. This beneficial effect could not be replaced by collecting semen in egg yolk free diluent and could therefore not be attributed to mere seminal plasma dilution. However, this simple action of sperm “coating”, which was obvious after 4 days of storage, was not efficient in preventing the rapid decline in the percentage of motile spermatozoa. The reduction in sperm movement is probably the result of mitochondrial damage because sperm motility is associated with healthy mitochondria (Donnelly et al., 2000). This implicates also that the motility apparatus was not protected by “coating”, which was confirmed by the finding that mitochondrial injury, was greater than that of nuclear DNA. This also corroborates with the fact that a number of crucial events in apoptosis commence in the mitochondria and could be detectable before DNA fragmentation occurs (Donnelly et al., 2000).

Coating of spermatozoa can therefore be used to store liquid semen in a simple salt solution without any further addition of membrane protective agents. In our hands, this leads to a significant higher recovery (55%) of progressively motile and membrane- intact spermatozoa with a high mitochondrial membrane potential selected after density gradient centrifugation. The oocyte penetration potential of these superior “coated” spermatozoa

became obvious by their significantly higher oocyte penetration rate, which exceeded that of "control" spermatozoa with 57%.

Unexpectedly, after 4 days of storage no improvement on sperm characteristics and even a decline in oocyte penetration rate was observed when "coated" spermatozoa were stored in Hepes-TALP at pH 6. Under these conditions, a decline in mitochondrial membrane potential was observed without a corresponding decrease in membrane and DNA integrity. This damage to the regulatory mechanisms responsible for motility, leads to a very low concentration (5%) of superior spermatozoa obtained after Percoll®-centrifugation, whereas most of the spermatozoa were recovered at the interface of the 45 and 90% Percoll®-layers and were nonmotile. It is difficult to speculate to the causes of this finding since it is known that egg yolk has a constant pH of about 6.0, which normally does not change much during storage.

Our results indicate that sperm "coating" seems to reduce storage-dependent ageing processes of spermatozoa stored in a simple salt solution during 4 days. The oocyte penetration rate (80%) was preserved for up to 4 days at RT, but decreased to 50% at day 6. These rates were comparable to the oocyte penetration rates after aerobic sperm storage into CAPROGEN® commercial diluent supplemented with 5% egg yolk (Livestock Improvement Corporation, Hamilton, New Zealand) (88% after 3 days and 52% after 7 days) (Krzyzosiak et al., 2001). The decrease in oocyte penetration rate after storage for up to 6 days could be explained by the fact that all spermatozoa were recovered at the interface of the 45 and 90% Percoll®-layers, indicating that less motile spermatozoa were available for in vitro oocyte penetration. Therefore, it was investigated whether the replacement of Hepes-TALP by Triladyl®-diluent could improve sperm survival. Low-density lipoproteins of egg yolk bind firmly with the sperm plasma membrane and stabilize this membrane both during storage of spermatozoa at RT and during cold shock (Vishwanath et al., 1992). Salamon and Maxwell (2000) have also shown that egg yolk reduces the loss of acrosomal enzymes and prevents degenerative changes in the acrosome during liquid storage. This was confirmed by the fact that in our study, no decrease in oocyte penetration was found after 6 days of storage in Triladyl®-diluent.

Another important factor that affects sperm function after ejaculation is the temperature at which spermatozoa were stored after dilution. Care must be taken not to subject spermatozoa to cold shock, which occurs when freshly ejaculated semen is cooled quickly to temperatures below 16°C (Halangk et al., 1982). The evidence of the importance of protecting spermatozoa during cold shock was confirmed in the last experiment.

Previously stabilizing the membrane of spermatozoa before cooling was not sufficient to protect spermatozoa during storage in Hepes-TALP at 4°C. In this case, only the membrane integrity of “coated” spermatozoa was protected, without a corresponding protection of the regulatory mechanisms responsible for motility, leading to a sharp decline in motility after 4 days and in oocyte penetration rate after 6 days. Therefore, it remains necessary to protect the sperm plasma membrane, with low-density lipoproteins of egg yolk and glycerol (Vishwanath et al., 1992) through the critical temperature zones and during storage at 4°C for longer than 5 days.

In conclusion, spermatozoa “coated” during ejaculation for less than 5 minutes with Triladyl®-diluent yield significantly higher oocyte penetration rates than “control” spermatozoa after storage for 4 days in Hepes-TALP at pH 7. Further improvements were made by additional protection of spermatozoa during storage in egg yolk containing diluent. These results, in combination with a better understanding of the causes of sperm ageing, are very promising to improve preservation and oocyte penetrating ability of liquid bovine semen.

Acknowledgments

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6.

HORMONAL REGULATION OF BOVINE SECRETORY PROTEINS DERIVED FROM CAPUT AND CAUDA EPIDIDYMAL EPITHELIAL CELL CULTURES.

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De Pauw I., Goff A., Van Soom A., Verberckmoes S. and de Kruif A.

Abstract

The aim of this study was to investigate the effect of hormones (testosterone, dihydrotestosterone (DHT) and hydrocortisone) on protein secretion of caput and cauda epididymal epithelial cells cultured in principal cell medium (PCM). A confluent monolayer of caput and cauda epididymal epithelial cells was obtained in serum-containing PCM in presence or absence of hormones after 7 days of culture at 38.5°C (5% CO_2 in air). Protein secretion of epididymal epithelial monolayers incubated in serum-free PCM for 3 days was examined. The secreted proteins were separated by two-dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis (2D-SDS-PAGE). Comparison of the different protein patterns showed 61 spots in total of which 11 were only secreted in the presence of hormones, 3 appeared to show hormone-related changes and 25 were region specific. Most of these secreted proteins were low molecular weight acidic proteins. To obtain evidence of the epididymal origin of the secreted proteins, proteins present in caput and cauda epididymal plasma were analysed. In conclusion, our data indicate that hormones influence the synthesis of a number of caput and cauda epididymal proteins. Some of these proteins could be important for improving our understanding of sperm maturation and storage, and their acquisition of fertilizing ability.

Introduction

The mammalian epididymis is a complex organ where spermatozoa are matured and stored. The composition of luminal fluid varies along the length of the epididymis. This internal milieu is derived from rete testis fluid and is modified by the absorptive and secretory activity of the epididymal epithelium (Moore et al, 1990; Setchell et al, 1994). Specific secretions from epididymal principal cells associate with spermatozoa during their maturation and storage (Sylvester et al., 1991; Moore, 1996) and play a fundamental role in modifying the surface characteristics of spermatozoa in preparation for the events of fertilization. Most of these epididymal processes seem to be dependent on androgens, which are derived from the rete testis and the blood circulation. The most active regulators responsible for maintaining epididymal structure and other epididymal functions are testosterone and 5α-reductase metabolite dihydrotestosterone (DHT) (Robaire and Viger, 1995). These androgens play an important role in controlling the synthesis of a number of specific proteins by the epididymis (Brooks and Higgins, 1980; Jones et al, 1981, 1982). Some of these proteins are absorbed or integrated into the sperm plasma membrane during epididymal transit. Glycoproteins are the most prominent of these macromolecules (Bongso and Trounson, 1996). On the other hand several sperm plasma membrane proteins of testicular origin are lost or altered during epididymal transit of the spermatozoa. Because the regulation of epididymal epithelial functions and their effects on spermatozoa are still poorly understood, cell cultures of epididymal epithelial cells can provide useful information on epididymis and spermatozoal interaction as evidenced from studies in human and laboratory animals (Moore et al, 1986, 1992; Bongso and Trounson, 1996; Moore, 1996; Akhondi et al, 1997). Akhondi and Moore (1993) have shown that principal cells can remain polarized and continue to secrete proteins for many days, sometimes weeks, in the presence of androgens and there is seldom fibroblast overgrowth. Effect of androgens on protein synthesis by the epididymis of laboratory animals and humans has already been demonstrated by many investigators, as summarized by Holland and Orgebin-Crist (1988). However, similar data on the bovine epididymis are lacking.

Recently, prolonged cultures of epididymal cells have also been established in cattle (Gagnon et al, 2000; Reyes-Moreno et al, 2000). Moreover, it has been shown that motility of frozen-thawed spermatozoa was partially preserved for 48 h after co-culture with either caput, corpus or cauda epididymal cells, whereas conditioned medium (Gagnon et al, 2000) or bovine epididymal plasma (Reyes-Moreno et al, 2002) was only effective in

preserving sperm motility for 6 h. It was concluded that epididymal epithelial cells secrete one or more beneficial compounds, which prolong sperm viability. This compound must be a common factor present in epididymal plasma and secreted by epididymal epithelial cells cultured in vitro. A number of epididymal proteins may be involved with the protection of ejaculated spermatozoa during in vitro storage (Reyes-Moreno et al, 2002). The aim of our study was to investigate which proteins:

1) are influenced by the presence of testosterone, dihydrotestosterone and hydrocortisone in the culture medium,
2) are common to conditioned media of caput and cauda epididymal epithelial cell cultures,
3) are common to conditioned media of caput or cauda epididymal cell cultures, and epididymal plasma.

Materials and Methods

Medium

Principal cell medium (PCM) consisted of RPMI-1640 medium (Life Technologies, Gibco BRL products (Merelbeke, Belgium)) supplemented with 10% fetal calf serum (FCS), 100 nM insulin, 1 mM sodium pyruvate, 5 µg/ml transferrin, 200 nM hydrocortisone, 200 nM testosterone, 1 µM dihydrotestosterone, 1 µg/ml retinol and 50 µg/ml gentamycin. Testosterone and DHT were used because they control the synthesis and secretion of a number of specific proteins by the epididymis and maintain the epididymal stucture and functions (Robaire and Viger 1995). Hydrocortisone promotes cell attachment (Ballard and Tomkins 1969) and cell proliferation (Guner et al. 1977). Principal cell medium without hydrocortisone, testosterone and DHT was used as control.

Collection of caput and cauda epididymal plasma

Epididymides of beef bulls aged 2-2.5 years were collected at a local slaughterhouse. After removing superficial blood and tissue fluid contamination, the pressure in the epididymal ducts was increased by clamping two pincers on the proximal and distal part of the caput and cauda epididymidis. Incisions were made in the connective tissue avoiding small blood vessels. Epididymal plasma was oozed out, aspirated into a fine pipette and transferred into a small tube. After 5 min of centrifugation at 3.214 x g, the

epididymal plasma was removed, checked visually for the absence of spermatozoa and frozen at - 20°C.

Epididymal epithelial cell culture

The epididymal epithelial cell culture was prepared by a modified protocol according to Moore et al (1986). The epididymal tissue was obtained from bulls slaughtered in a local slaughterhouse. On arrival in the laboratory, the caput and cauda epididymidis from one bull were dissected free of the testis, fat and connective tissue and washed in RPMI-1640 to remove blood. Both regions were minced in small segments of 1-2 mm using scissors and placed in PCM (Moore et al, 1992). Spermatozoa within tubule segments were teased out using forceps. The tubule segments were then incubated in PCM containing 1.5 mg/ml collagenase type II (Sigma, Bornem, Belgium) at 38.5°C in 5% CO_2 in air for 2 h. After enzymatic digestion, tubule segments were dissected free of surrounding collagen with a needle, washed again in PCM to remove remaining spermatozoa and transferred to fresh medium. They were slit open longitudinally and cut into small fragments. These fragments were prepared from both caput and cauda epididymidis and cultured separately in PCM supplemented with fibronectin (2µl/ml) (Sigma, Bornem, Belgium) and in PCM without hormones, and supplemented with fibronectin (2µl/ml) (control) at 38.5°C and 5% CO_2 in air. The culture medium was changed every other day. The fragments formed irregular contiguous spheres of epithelial cells with apical surface facing outwards and remained free floating in culture during the first days. These spheres became attached to the dish and the epithelial cells spread out (Moore et al, 1992). Attached explants were carefully removed with a 26-gauge needle connected to a 1-ml syringe. Epithelial cells continued to divide forming a monolayer after 5 to 7 days of culture. The medium was then replaced by serum-free PCM with or without hormones. After 3 days of incubation, cultures were examined for epithelial cell detachment by means of inverted light microscopy. Epithelial and fibroblast cell concentration of the monolayer was examined by immunohistochemistry. The medium was collected from the different groups and stored at -20°C until analysis by two-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (2D-SDS-PAGE).

Immunohistochemistry

Monolayers cultured on glass coverslips are rinsed with phosphate-buffered saline (PBS), fixed for 10 min in acetone and air-dried. After rinsing the slides 3 times with PBS, the cells were incubated for 2 h with an anticytokeratin antibody (Keratin Pan Ab-1, NeoMarkers, CA, USA) diluted 1/500 in PBS at 37°C, to establish the proportion of epithelial cells (Henriksen et al, 1990). Followed by washing the epithelial cells for 10 min in PBS and incubating with a biotin-conjugated goat anti-mouse immunoglobulin antibody at RT for 30 min. After washing the slides in PBS for 10 min, the cells were incubated with Strept-ABComplex/horseradish peroxidase at RT for 30 min. Horseradish peroxidase activity was visualised by incubating the slides with 3,3'-diaminobenzidine tetrahydrochloride (DAB tablets, Sigma) 0.5 mg/ml Tris-HCl buffer, pH 7.6 containing 0.02% hydrogen peroxide for 10 s resulting in brown staining. After washing, cells were counterstained with Mayer's Hemaluin solution (VWR International, Leuven, Belgium), washed, coversliped and viewed with light microscopy (Leica DMR, Van Hopplynus N.V., Brussels, Belgium). Another replicate of epididymal cultures was probed with antibody against vimentin (Vimentin Ab-2 (V9), NeoMarkers, CA, USA) to establish the proportion of fibroblasts. The epithelial and fibroblast cell concentration was measured with the Image Database Program of Leica (Van Hopplynus NV, Brussels, Belgium).

Two-Dimensional Gel Electrophoresis

Before separation and analysis of the proteins by means of 2D-SDS-PAGE, the proteins of the conditioned media were concentrated using Ultrafree-15 concentrators (5000 MW cutoff; Millipore). The protein concentration of the different samples was measured using the Bio-Rad protein assay reagent. Proteins were then added to IPG buffer (8M Urea, 2% CHAPS, 0.5% IPG Buffer (pH 3-10), bromophenol blue, 65 mM Dithiothreitol (DTT)) to give a final concentration of 2µg/100µl IPG buffer. The separation in first dimension was carried out using immobiline DryStrips that had been rehydrated in 250 µl of the sample/IPG buffer solution (5 µg total protein) for at least 10 h in an Immobiline™ Drystrip Reswelling Tray. The samples were then separated on a MultiPhore II flatbed system for 16 h at 15°C. The voltage was 300 V for the first 3 h, from 300 to 2000 V during the following 5 h, and finally 8 h at 2000 V. Before the second dimension was performed, the dry strips were first equilibrated for 10 min in Equilibration solution 1 (0.5 M Tris/HCl (pH 6.8) containing 0.36 g/ml urea, 10 mg/ml SDS, 2,5 mg/ml (DTT) and

26% glycerol) and another 10 min in Equilibration solution 2 (0.5 M Tris/HCl (pH 6.8) containing 0.36 g/ml urea, 10 mg/ml SDS, 45 mg/ml iodoacetamid and 26% glycerol). The second dimension was performed after placing the strips on Pharmacia ExcelGel® XL SDS 12-14 using the MultiPhore II flatbed system for 3-4 h at 15°C. After running the gels, they were immediately immersed in fixing solution (50% methanol, 10% acetic acid in water) and stained with silver nitrate (Silver Staining Kit, Amersham Pharmacia Biotech AB, Baie d'Urfé, Canada). Gels were compared and the molecular weight (MW) and isoelectric point (pI) of the proteins were calculated using the Phoretix 2-D SDS PAGE Analysis Software (Phoretix, Newcastle-upon-Tyne, England).

This experiment was repeated with three different caput and cauda epithelial cell cultures. A representative protein pattern of each culture condition was obtained by comparing scans of three gels of each culture, and only spots present on the three gels were taken into account. The concentration of each spot was expressed as its spot volume, which is the product of the area of the spot and its total optical density. Differences in spot density between cultures was determined by Students t-test.

Results

Formation of cell cultures

A confluent monolayer of caput and caudal part of the epididymis was successfully obtained after 5 to 7 days of culture in serum-containing PCM at 38.5°C in 5% CO_2 in air with or without the presence of hormones. From day 7 until day 10 the medium was replaced by serum-free PCM with or without hormones. Under all cell culture conditions, there was no epithelial cell detachment for the first 10 days in culture. Primary cell cultures of caput and cauda epithelial cells can be maintained for up to 10 days without substantial overgrowth of fibroblasts, as determined with antibodies to vimentin. More than 90% of the cells in the monolayer were epithelial cells as identified by the presence of cytokeratin (results not shown). No significant difference in mean epithelial cell concentration of 3 replicates was observed between hormone-containing cultures (caput = 398040 epithelial cells/1.77 cm²; cauda = 346680 epithelial cells/1.77 cm²) and hormone-free cultures (caput = 369792 epithelial cells/1.77 cm²; cauda = 333840 epithelial cells/1.77 cm²) (ANOVA, SPSS 10.0 for Windows).

Protein analysis using 2-D SDS PAGE

Comparison of the different protein patterns showed 61 proteins secreted by epididymal epithelial cell cultures. Some of these proteins were induced, down-regulated or the concentration was increased or decreased in the presence of hormones (Table 1). Forty-seven of these proteins were also observed in caput or cauda epididymal plasma (Figure 1).

Table 1. The effect of hormones (200 nM hydrocortisone, 200 nM testosterone, 1 µM dihydrotestosterone) on the protein concentration and on the induction or down-regulation of proteins secreted by caput or cauda epididymal epithelial cell cultures.

	CAPUT	CAUDA
	Spot number	
Increased concentration	-	4
Decreased concentration	-	10, 50
Unaltered concentration	1, 2, 7, 8, 10, 11, 12, 13, 14, 16, 19, 34, 41, 42, 43, 44, 46, 48, 49, 50, 53, 58, 60, 63	1, 2, 6, 8, 11, 12, 13, 14, 16, 17, 21, 22, 29, 30, 38, 40, 41, 42, 43, 44, 46, 48, 49, 53, 54, 58, 60
Induced proteins	15, 23, 31, 36, 38, 45, 64	3, 23, 25, 27
Down-regulated proteins	4, 17, 18, 28, 30, 32, 37, 40, 47, 51, 52, 55, 56, 59, 61, 62	5, 7, 9, 18, 24, 26, 32, 33, 34, 35, 47, 51, 52, 56, 59, 61

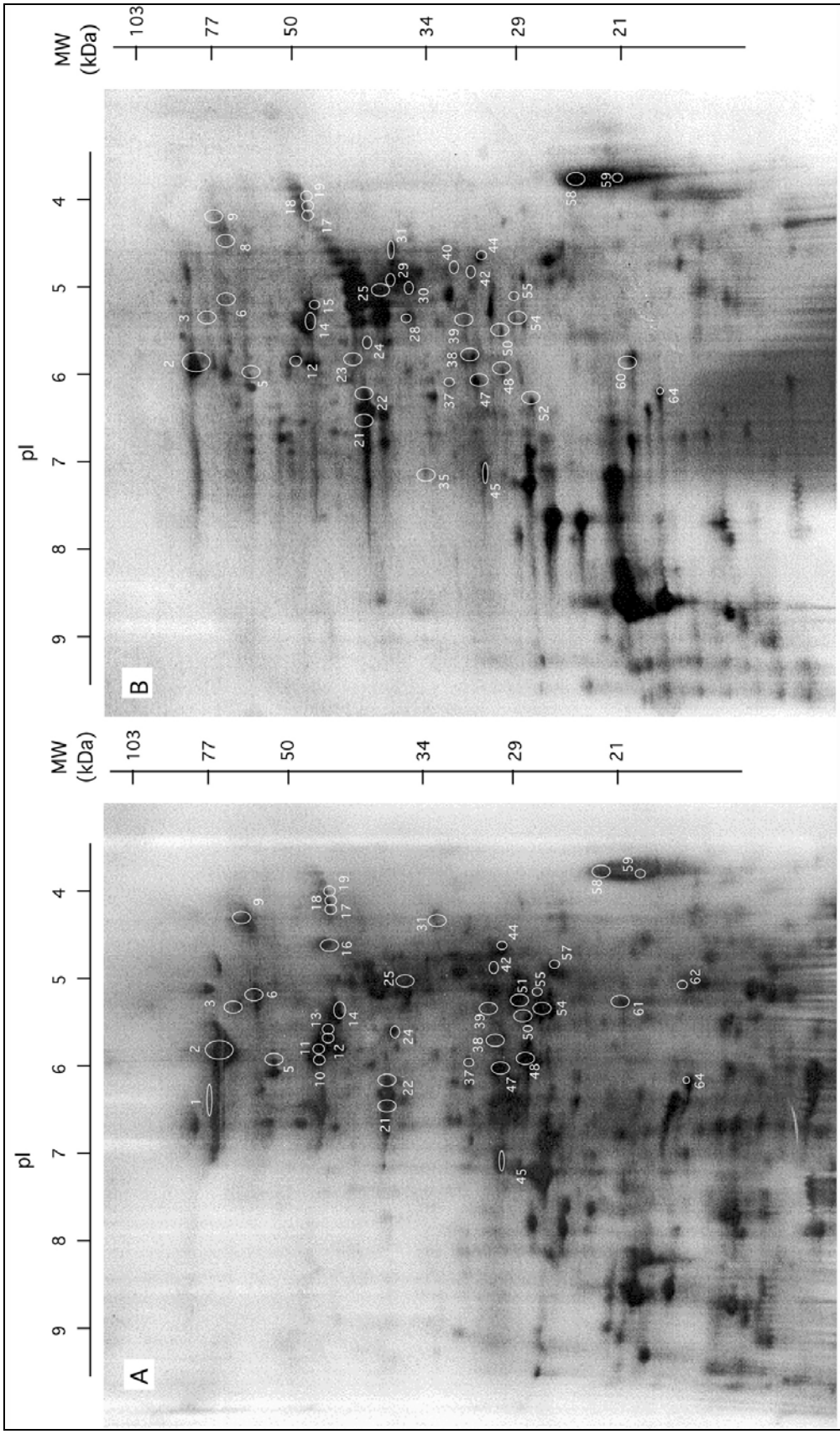

Figure 1. Two-dimensional SDS-PAGE separation of proteins of epididymal plasma. Phoretix 2D Advanced software was used to detect and compare protein spots. The marked spots represent proteins that correspond to those found in the medium after culture of caput or cauda epithelial cells (see Figures 2 and 3) (A) Plasma from the caput region (B) Plasma from the cauda region.

The differences in protein secretion from caput epithelial cells cultured in presence or absence of hormones are shown in figure 2. At least 47 proteins were secreted into the conditioned media of which 31 were also present in caput epididymal plasma. Most of them had an acidic pI in the range of 4 to 6.5. Twenty-four proteins were secreted in both control and hormone-containing culture. There was variation in spot intensity between experiments but no significant effect of treatment on the amount of these secreted proteins was observed. However, the secretion of 23 proteins was preferentially regulated by hormones. Seven proteins (spot number 15, 23, 31, 36, 38, 45 and 64) were induced, these proteins were not secreted in the absence of hormones, and 16 proteins (spot number 4, 17, 18, 28, 30, 32, 37, 40, 47, 51, 52, 55, 56, 59, 61 and 62) were down-regulated by hormone treatment.

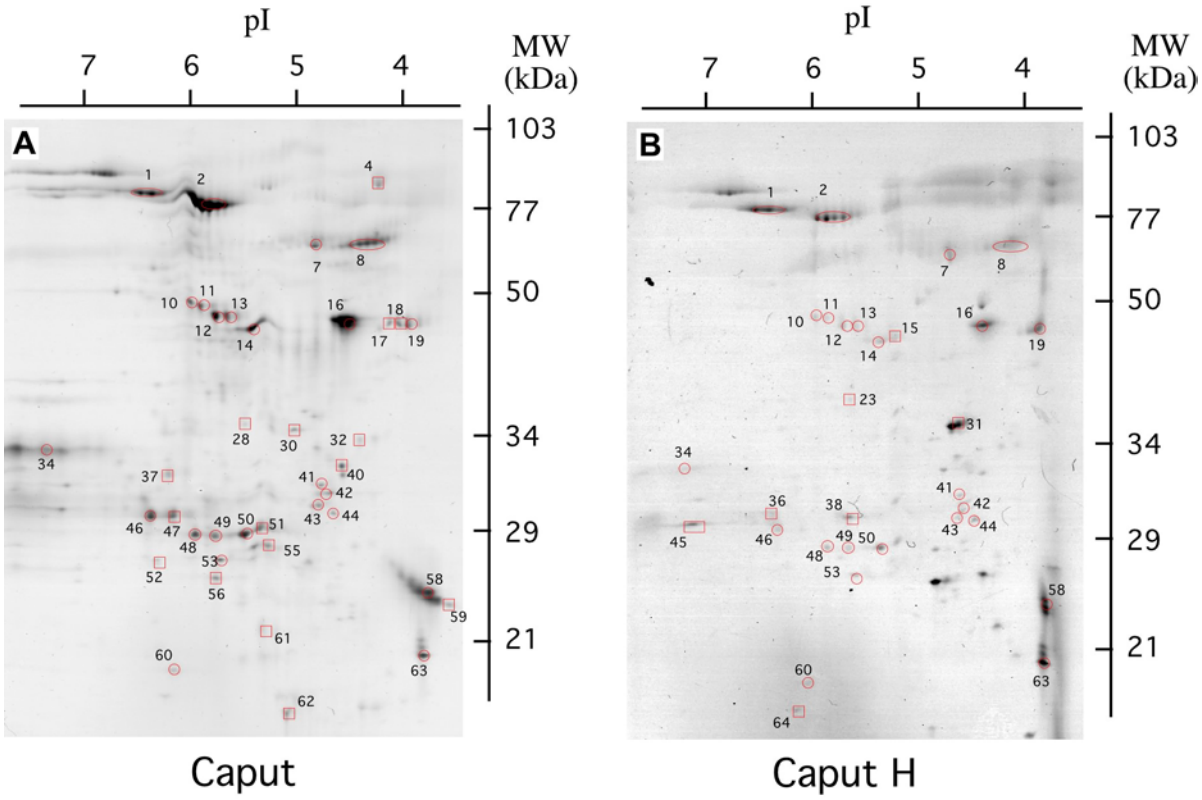

Figure 2. Two-dimensional SDS-PAGE separation of proteins secreted from caput epididymal cells. Phoretix 2D Advanced software was used to detect, compare and calculate the volume of protein spots. Only spots found on three different replicates were labeled and all spots were numbered sequentially starting with the highest MW. Gels are shown for epithelial cells cultured in PCM (Caput), and epithelial cells cultured in PCM + hormones (200 nM testosterone, 1 µM dihydrotestosterone and 200 nM hydrocortisone) (Caput H). Spots marked with a circle are common to both control and hormone-treated cultures, and spots marked with a square are found only in control or only in hormone-treated cultures

The effect of hormones on protein secretion from cauda epididymal epithelial cells is presented in Figure 3. At least 50 proteins were secreted into the conditioned media of which 30 were also observed in cauda epididymal plasma. Of the 30 proteins found in both control and hormone-treated samples, the intensity of 3 proteins was significantly ($P < 0.05$) altered by hormones. The intensity of protein 4 was increased in the presence of hormone whereas that of proteins 10 and 50 was decreased. The secretion of 20 proteins, with a pI in the range of 4 to 6.5, was regulated by hormone treatment: 4 proteins (spot number 3, 23, 25 and 27) were induced by hormones and 16 proteins (spot number 5, 7, 9, 18, 24, 26, 32, 33, 34, 35, 47, 51, 52, 56, 59 and 61) were down-regulated.

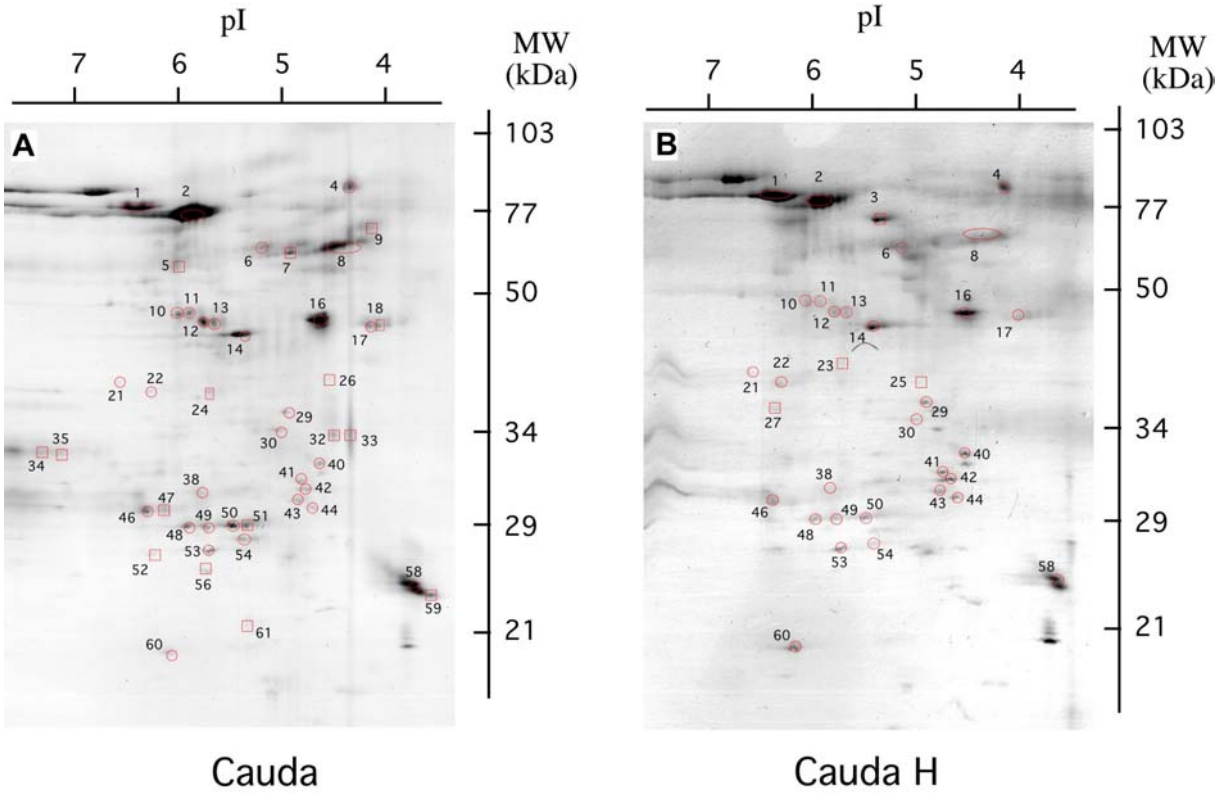

Figure 3. Two-dimensional SDS-PAGE separation of proteins secreted from cauda epididymal epithelial cells in culture. Phoretix 2D Advanced software was used to detect, compare and calculate the volume of protein spots. Only spots found on three different replicates were labeled and all spots were numbered sequentially starting with the highest MW. Gels are shown for epithelial cells cultured in PCM (Cauda) and epithelial cells cultured in PCM + hormones (200 nM testosterone, 1 µM dihydrotestosterone and 200 nM hydrocortisone) (Cauda H). Spots marked with a circle are common to both control and hormone-treated cultures and spots marked with a square are found only in control or only in hormone-treated cultures

Differences in protein composition secreted by epididymal epithelial principal cells from caput versus cauda epididymidis were also observed (Figure 4). Of the 61 proteins that were consistently present, 11 proteins (spot number 15, 19, 28, 31, 36, 37, 45, 55, 62, 63, and 64) were only secreted by caput epithelial cells while 14 proteins (spot number 3, 5, 6, 9, 21, 22, 24, 25, 26, 27, 29, 33, 35, and 54) were unique to cauda epithelial cell cultures. Thirty-six proteins were secreted by both caput and cauda epididymal epithelial cells.

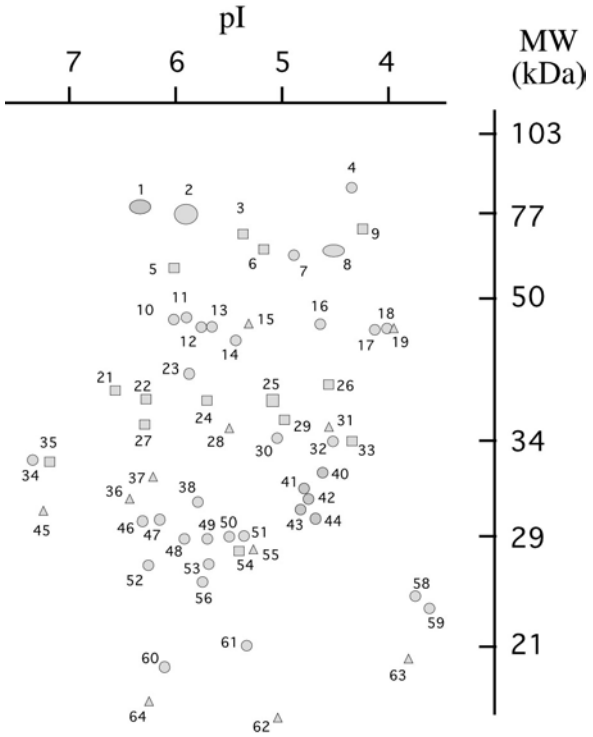

Figure 4. General master pattern summarizing the total number of protein spots from media of control or hormone-treated caput and cauda epididymal epithelial cells. Spots marked with a triangle are secreted only in control or hormone-treated caput epididymal epithelial cell cultures, spots marked with a square are secreted only in control or hormone-treated cauda epididymal epithelial cell cultures, and spots marked with a circle are common to both caput and cauda cell cultures

Discussion

In cattle, little is known by which processes epididymal epithelium and its secretions can maintain sperm viability in vitro. These processes appear to be mediated by proteins secreted by epididymal mucosa under the influence of androgens. Although epididymal cell culture or even conditioned medium (both in the presence of androgens) can be used to improve bovine sperm survival and/or motility in vitro (Gagnon et al, 2000;

Reyes-Moreno et al, 2000), this approach is no practical alternative for the development of a diluent for prolonged storage of liquid bovine semen.

Evidence for viability and normal differentiation of our caput and cauda epididymal epithelial cell cultures was given by the fact that both regions displayed the same capacity to form a confluent monolayer of epithelial cells in PCM and remained viable in vitro for at least 10 days. In contrast with the results of Gagnon et al (2000) and Moore et al (1992), no signs of cell degeneration, reduced growth or contamination with fibroblast were apparent in caput and cauda epididymal epithelial cell culture in the absence of hormones. The cultured epithelial cells exhibited less than 10% contamination with other cell types, as examined by staining with cytokeratin. This percentage was similar with the observations made by Gagnon et al (2000).

To improve our insight in the molecular mechanism of sperm protection, we have focused our attention on proteins present in conditioned media from epididymal cultures of caput or cauda epithelial cells, in the presence or absence of hormones. We have shown that 66% and 60% of the proteins present in respectively caput or cauda conditioned media correspond to proteins secreted in caput or cauda epididymal plasma, which confirms their epididymal origin. Proteins present in conditioned media while absent in epididymal plasma could correspond to proteins that are integrated into the sperm plasma membrane during epididymal transit, or which are degraded or reabsorbed rapidly after secretion.

The majority of secreted proteins had molecular masses between 20 and 80 kDa and an acidic pI value between 4 and 7. Besides the hormone dependency of protein synthesis by epididymal cells, substantial differences in protein patterns from caput and cauda epididymal regions could be detected (Figure 4). In addition to proteins that were induced uniquely by hormones, secretion of only a few proteins was either enhanced or reduced by these hormones.

The precise function of these secreted proteins in sperm maturation and storage processes remains to be established. Indirect evidence for a function can come from their relative abundance and from their site-specific expression along the epididymal duct. A further approach could be the identification of some major epididymal proteins by means of protein microsequencing, recombinant DNA techniques, use of specific antisera (Syntin et al, 1996), or by investigating their sequence similarities to proteins or protein families of known functions, and their immunolocalization on the sperm surface (Kirchhoff, 1998). The concentration of a protein present in epididymal plasma from different regions is not necessarily related to its level of secretion (Syntin et al, 1996). Proteins present at low

concentration in epididymal plasma may be those that are degraded, reabsorbed or integrated into the sperm membrane (Syntin et al, 1996) and thus are possibly of greater interest than more abundant proteins. This is in contrast with the presence of proteins in conditioned media, where most important proteins were probably those most abundantly secreted because no reabsorbing or integrating into the sperm membrane could occur. Proteins found uniquely in caput may be important in influencing specific maturational changes in spermatozoa as they transit the duct, whereas cauda-specific proteins may be important for the storage of spermatozoa. Such proteins may also be involved in regulating the structural and functional integrity of the epididymis itself.

Our results are consistent with the findings of Reyes-Moreno et al (2002), however no protein identification was done in our study. Reyes-Moreno et al (2002) have characterized 5 bovine proteins, secreted in cauda epididymal plasma, which could play a role in sperm protection in vivo. One protein identified as beta-adrenergic receptor kinase 2 corresponds probably to protein 14 in the present study. Protein spots 10 and 11 (of about 48 kDa) may correspond to antithrombin-III and fibrinogen gamma-B chain found by Reyes Moreno and coworkers (2002). In our study, these proteins were secreted in both regions of the epididymis. Beta (36-kDa spot) chain of clusterin, which corresponds to protein number 25 in our study, was only secreted by cauda epididymal epithelial cells in the presence of androgens. In vivo studies in the bull have shown similar regional secretion of the beta chain of clusterin (Howes et al., 1998).

It is concluded that androgens are involved in the regulation of protein synthesis by the epididymis. Identification and localisation of these proteins on spermatozoa could provide useful information on how spermatozoa can survive and preserve their metabolic quiescent state in the epididymis. To fully understand the regulation of the epididymis, a combination of molecular interactions between sperm surface and epididymal epithelium, ionic composition and physiological conditions of the epididymal plasma must also be taken into account.

Acknowledgements

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7.

SPERM BINDING TO EPITHELIAL OVIDUCT EXPLANTS IN BULLS WITH DIFFERENT NON-RETURN RATES INVESTIGATED WITH A NEW IN VITRO MODEL

Biology of Reproduction 2002; 67: 1073-1079

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Abstract

A new in vitro method was developed for analyzing the capacity of spermatozoa to bind to oviductal epithelium to determine whether this binding capacity could be used to predict non-return rates (NRR). Sperm binding was evaluated by counting 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1)-labeled spermatozoa attached to oviduct explants and by measuring the surface area of oviduct explants by means of an image analysis program. Hepes + Tyrode albumin lactate pyruvate (TALP) was a more useful medium than in vitro fertilization (IVF)-TALP, TCM-199 medium + 10% fetal calf serum, and TCM-199 medium alone for the investigation of sperm binding to oviduct explants. Oviduct explants with a surface area of < 20 000 µm² provided more consistent results than did explants with a surface area of > 100 000 µm². A positive association was found between the $\log_e$-transformed number of spermatozoa bound to 0.1 mm² oviductal epithelium and the NRR of the respective sires when evaluated after 24 h of sperm-oviduct incubation, provided that the membrane integrity of the sperm sample was > 60%. Determination of the capacity of spermatozoa to bind to oviduct explants could become a reliable in vitro method for predicting the NRR of a given sire.

Introduction

In most mammals, ejaculated spermatozoa pass through the female reproductive tract to form a functional sperm reservoir in the lower segment of the oviduct (Yanagimachi, 1994). In cattle, it takes about 6-8 h before enough spermatozoa have reached this sperm reservoir to ensure fertilization (Hunter and Wilmut, 1982). Spermatozoa attach to the apical plasma membrane of the ciliated and secretory epithelial cells (Suarez et al., 1990). This attachment is mediated by fucose recognition (Lefebvre et al., 1997). Other factors that could be involved in the establishment of a sperm reservoir are impedance of sperm movement by oviductal mucus (Suarez et al., 1997) and low patency of the oviduct due to edematous mucosa and tightly contracted myosalpinx (Hunter, 1997).

The reservoir acts to ensure that enough fertile spermatozoa are available in the oviduct when ovulation occurs. Bovine spermatozoa can remain arrested in the isthmus for ≥ 18 h and only detach from the epithelium near the time of ovulation (Hunter and Wilmut, 1984). This prolonged sperm survival is especially important in species with longer estrous periods, such as the horse (Day, 1942), but might also be of interest for cattle, which are inseminated early in estrus. If a bull is unable to populate the sperm reservoir with a sufficient number of spermatozoa or for a sufficient period of time, fertility could be adversely affected. The field fertility of a given bull is expressed by the non-return rate (NRR), which is defined as the proportion of cows that were inseminated and did not return for another service within 56 days (den Daas et al., 1998). Differences probably exist among bulls in their capacity to establish a sperm reservoir after mating or insemination, but it is difficult to detect these differences after matings and collection of oviducts because the number of spermatozoa reported to reach the oviduct in vivo has differed considerably among studies and within experiments (Parker et al., 1975; Mburu et al., 1996; Suarez et al., 1997).

To determine whether the capacity to establish a reservoir is indicative of fertility, we searched for an in vitro approach to study the capacity of spermatozoa to bind to oviductal epithelium. In vitro sperm membrane integrity can be extended by coincubating spermatozoa with oviductal epithelium (Pollard et al., 1991). To investigate this sperm binding in more detail, we needed a reliable method to determine the number of spermatozoa attached to oviduct explants. The aim of this study was to set up a model to quantify sperm binding to oviduct explants by combining fluorescent staining of

spermatozoa and image analysis. After standardizing, this model was tested to evaluate whether sperm binding density could be used to predict in vivo bull fertility. For this aim, sperm binding density to oviduct explants was assessed in sires with known NRRs, which are indicative for in vivo fertility.

Materials and Methods

Media

Chemicals and media were obtained from Sigma (Bornem, Belgium) and Gibco Invitrogen Corporation (Merelbeke, Belgium).

The following media were used in this study:

Hepes-buffered Tyrode albumin lactate pyruvate (TALP) medium contained 114 mM of NaCl, 3.1 mM of KCl, 0.3 mM of NaH2PO4, 2.1 mM of CaCl2, 0.4 mM of MgCl2, 2 mM NaHCO3, 0.2 mM sodium pyruvate, 10 mM sodium lactate, 10 µg/ml gentamycin sulphate, 10 mM Hepes and 3 mg /ml BSA.

In vitro fertilization (IVF) medium contained TALP without Hepes supplemented with 25 mM $NaHCO_3$, 0.2 mM sodium pyruvate, 10 mM sodium lactate, 10 µg/ml gentamycin sulphate, and 6 mg/ml fatty acid-free BSA (Sigma). Heparin was omitted because it would prevent capacitation and heparin-induced sperm release from oviduct explants (Bosch et al., 2001; Talevi and Gualtieri, 2001).

Modified bicarbonate buffered TCM-199 was supplemented with 0.2 mM sodium pyruvate, 0.4 mM glutamine, 50µg/ml gentamycin sulphate and 2.5 µg/ml fungizone. Fetal calf serum (FCS: 10%) was added as noted.

Media were passed through sterile 0.22 µm Acrodisc® Syringe low protein binding filters (Millipore Corp., New Bedford, MA) before being used.

Preparation of spermatozoa

For standardization of the model to study sperm binding to oviduct explants in vitro, fresh and frozen-thawed semen of a 2-yr-old Red Pied bull were used.

To study the relationship between NRR and sperm binding to oviduct explants, we used frozen-thawed semen from 10 Holstein Friesian bulls with known fertility (expressed

as 56-day NRR) varying from 52.8% to 69.9%. The difference between the highest and the lowest NRR was 17%. This was the maximum range available from the Artificial Insemination (AI) centre. The NRRs were based on a total of 1884 first inseminations with frozen-thawed semen with a minimum of 163 AIs per bull. Straws were generously supplied by VRV (Flemish Cattle Breeding Association, Belgium). Artificial inseminations were performed by experienced veterinarians in dairy herds during fall and winter of 2000-2001. Uncorrected averages of NRRs were used, but bias was kept to a minimum by using sires with comparable numbers of services, which were used in the winter period (less use of natural breeding). Semen was processed for freezing as described by den Daas et al., 1998. Frozen-thawed semen obtained from the same ejaculate was used both for the investigation of sperm binding to oviduct explants as for the determination of NRR. Two straws of frozen semen were thawed in a water bath at 37°C for at least 30 s and washed twice in 5 ml of Hepes-TALP solution by centrifugation (720 x g, 10 min) at room temperature. After removing the supernatant, the sperm concentration was measured with a Bürker chamber. The final sperm concentration added to the oviduct explants was 10^6 spermatozoa/ml. This concentration was similar to the concentration used in related studies (Lefebvre and Suarez, 1996; Gualtieri and Talevi, 2000). The progressive motility was subjectively assessed by visual estimation under a light microscope (Leica DMR, Van Hopplynus NV, Brussels, Belgium; magnification 200 x) equipped with a stage warmer (37°C). The nucleic stains SYBR®-14 and propidium iodide (PI) (LIVE/DEAD® Sperm Viability Kit; Molecular Probes, Leiden, The Netherlands) were used for analyzing the membrane integrity of spermatozoa just before coincubation with the oviduct explants.

Collection and processing of oviducts for culture

Bovine oviduct explants were collected and prepared according to a modification of the procedure of Madison et al (1991). Oviducts were transported to the laboratory in physiological saline (0.9% NaCl) on ice. Upon arrival, they were dissected free of the surrounding tissues and rinsed in Hepes-TALP. The dissected oviduct was cut open longitudinally with sterile scissors under a laminar flow hood and was lightly scraped with a sterile scalpel to collect epithelial tissue. Sheets of epithelial cells were transferred to a 15-ml tube containing 5 ml Hepes-TALP. After initial sedimentation, the supernatant was removed, and 5 ml of fresh Hepes-TALP was added to the pellet. The same volume was removed again after the second sedimentation. The last washing was performed in

modified TCM-199-medium + 10% FCS. The supernatant was removed, and the cell sheets were disaggregated into smaller pieces by passing once through a 21-gauge needle attached to a 1-ml syringe.

Two hundred microliters of sedimented oviduct explants was added to 5 ml modified TCM-199-medium + 10% FCS and cultured overnight in a 50-ml flask. After overnight culture, the oviduct cells had formed clumps or worms with beating cilia. The medium containing the oviduct explants was transferred to a 15-ml tube. After sedimentation, the supernatant was removed, and 50 µl of the oviduct explants was added to 250 µl of the prepared semen sample and 200 µl of medium in a four-well culture plate.

Quantification of sperm binding to oviduct explants

The number of spermatozoa bound to oviduct explants was determined using 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1; Molecular Probes) to stain mitochondria. This fluorophore exhibits potential-dependent accumulation in mitochondria and can reversibly change its emission from green to red with increasing transmembrane electrical potential (Garner and Thomas, 1999). JC-1 was combined with the classical dead cell stain PI to identify membrane-damaged spermatozoa (Garner et al., 1986) (Figure 1). Density of bound spermatozoa on one side of the oviduct explant was determined after 30 min, 24 and 48 h by means of fluorescence microscopy performed by using a Leica DMR microscope equipped with an excitation filter of 450 - 490 nm from a 100 W mercury lamp and examined at a magnification of 100 x (explants with a surface area of > 100 000 µm²) or at a magnification of 400 x (explants with a surface area of < 20 000 µm²). The surface of the oviduct explant was measured with the Image Database Program (Leica, Van Hopplynus NV, Brussels, Belgium).

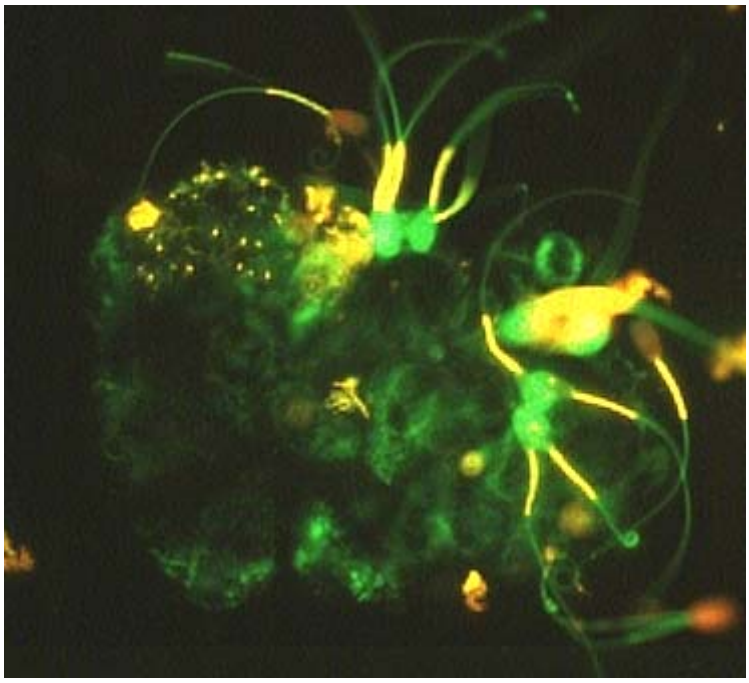

Figure 1: Fluorescence image of spermatozoa bound to oviduct explant stained with JC-1, which labels the mitochondria of spermatozoa orange and that of oviduct cells green, and with PI, which stains the heads of membrane-damaged spermatozoa red (magnification x 400)

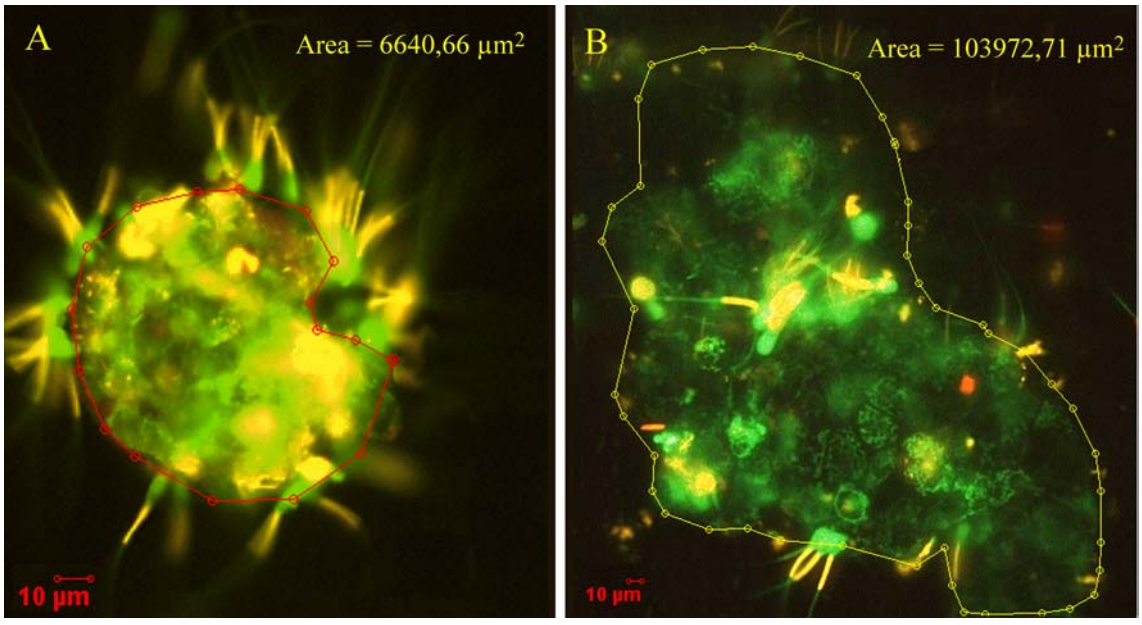

Figure 2: Bull spermatozoa are bound to oviduct explants and labeled with JC-1 and PI. **A**) explant surface area < 20 000 µm² (× 400). **B**) explant surface of > 100 000 µm² (magnification × 100)

Experimental design

Experiment 1: Standardization of a model to study sperm binding to oviduct explants

Effect of media and sperm preservation method on binding of fresh and frozen-thawed spermatozoa to oviduct explants

Frozen-thawed and fresh spermatozoa from a Red Pied bull were washed and incubated separately with oviduct explants at a concentration of 10^6 spermatozoa/ml medium. Small and large sized oviduct explants were equally distributed between fresh and frozen-thawed spermatozoa.

The different media, Hepes-TALP, IVF-TALP, TCM-199 + 10 % FCS, and TCM-199 were incubated at 38.5°C in 5% CO_2 except for Hepes-TALP (38.5°C, in air). After 30 min, 24 and 48 h of coincubation, at least 10 oviduct explants were stained with JC-1 and PI for 15 min. The oviduct explants with bound spermatozoa were first transferred twice to fresh medium by means of a micropipette (Unopette Capillary Pipettes, Becton Dickinson, Franklin Lakes, NJ) to remove unbound spermatozoa and then placed on a glass slide and viewed under a Leica DMR fluorescence microscope (magnification x 100 or x 400) equipped with a stage warmer (37°C). The surface area of 10 oviduct explants per point of time and per medium was measured, and the number of spermatozoa bound to one side of the oviduct explant was calculated. The size of the explants varied between 9200 µm² and 137 219 µm². The number of spermatozoa bound to 0.1 mm² of explant surface was used as the parameter of binding capacity. The experiment was repeated three times. Each replicate was performed with oviduct explants from the same batch.

Effect of the size of oviduct explants on sperm binding

The binding of frozen-thawed spermatozoa to 10 oviduct explants with a surface area of < 20 000 µm² (Figure 2A) and to 10 oviduct explants with a surface area of > 100 000 µm² (Figure 2B) was compared after 30 min of coincubation. Sperm binding density was expressed as the number of spermatozoa bound to 0.1 mm² of oviduct epithelium. The experiment was repeated three times. Each replicate was performed with oviduct explants from the same batch.

Experiment 2: Relationship between NRR and capacity of spermatozoa to bind to oviduct epithelial explants

The optimized model was used to investigate whether there is a difference in capacity to bind to oviduct explants for frozen-thawed spermatozoa from 10 Holstein Friesian bulls with different NRRs (52.8-69.9%). The influence of the initial membrane integrity on the association between the $\log_e$ transformed number of spermatozoa bound to 0.1 mm² of oviduct epithelium and the NRR was also investigated. Membrane integrity of spermatozoa from each sperm sample was evaluated by fluorescence microscopy just before addition to oviduct explants (LIVE/DEAD® Sperm Viability Kit). This experiment was repeated 3 times.

Frozen-thawed spermatozoa were used for this experiment because all AIs were carried out with frozen-thawed spermatozoa. Only oviduct explants with a surface area of < 20 000 µm² (obtained by passing twice through a 26-gauge needle) were used. The mean values and ranges of size of oviduct explants were comparable for each bull.

Statistical analysis

The effect of different media and sperm preservation methods (fresh versus frozen-thawed) on capacity of spermatozoa to bind to epithelial oviduct explants was analyzed with the Mixed procedure of SAS version 8 (SAS Institute, Cary, NC). The outcome variable was expressed as the $\log_e$ transformed number of spermatozoa bound per 0.1 mm² oviduct explant. Explanatory variables were the media and the sperm preservation method. Both explanatory variables were included as class variables. Time was considered a repeated measure and batch a random effect.

The effect of the size (small = < 20 000 µm², large = > 100 000 µm²) of oviduct epithelial explants on sperm binding was analyzed by means of a two-sample t-test. Differences were considered significant at $P = 0.05$.

The effect of different bulls with given NNRs and membrane integrity rates on capacity of spermatozoa to bind to epithelial oviduct explants was analyzed with the Mixed procedure of SAS version 8. The outcome variable was expressed as the $\log_e$ transformed number of spermatozoa bound per 0.1 mm² of oviduct explant. Explanatory variables were the membrane integrity rate (%) and the NRR (%). Both explanatory variables were included as continuous variables. Time was considered a repeated measure, and bull was a

random effect. Regression lines of the number of spermatozoa bound to epithelial oviduct explants after 24 h against the NRR and for different membrane integrity rates (40%, 45%, … 85%) were calculated by using the ESTIMATE statement of the Mixed procedure.

Results

Experiment 1: Standardization of a model to study sperm binding to oviduct explants

An additional experiment was conducted to investigate whether the number of oviduct explants present in a 50-µl sample could affect the number of sperm binding to each oviduct explant. The number of oviduct explants present in a 50-µl sample taken from a mixture of both large and small oviduct explants or from small oviduct explants was counted. The results indicated that the mean number of oviduct explants was not significantly different between replicate samples (large and small oviduct explants: mean ± SEM = 182 ± 3, coefficient of variation = 0.06, n=10; small oviduct explants: mean ± SEM = 247 ± 5, coefficient of variation = 0.06, n=10).

Spermatozoa (10^6) were then coincubated with a culture of 200, 250, 275 and 300 small oviduct explants. The number of spermatozoa bound to 10 oviduct explants from each culture was counted after 30 min, 24 and 48 h. At each time point, no significant difference in the number of spermatozoa per 0.1 mm² of oviduct explant was observed when the number of oviduct explants ranged between 200 and 300 (ANOVA, Scheffé test; data not shown).

Effect of media and sperm preservation method on binding of fresh and frozen-thawed spermatozoa to oviduct explants

Significantly ($P < 0.05$; Table 1) more fresh spermatozoa were bound to oviduct explants incubated in Hepes-TALP or IVF-TALP than in the other media. Fresh spermatozoa remained bound to oviduct explants in Hepes-TALP in substantial numbers even after 48 h of coincubation. For frozen-thawed spermatozoa, differences in sperm-oviduct binding were observed between Hepes-TALP and TCM-199 with or without addition of 10 % FCS ($P < 0.05$; Table 1). The decrease in the number of frozen-thawed bound spermatozoa in Hepes-TALP differed ($P < 0.05$) from that in IVF-TALP over time. The binding capacity of fresh and frozen-thawed spermatozoa was not different ($P = 0.12$).

Because more spermatozoa remained bound to oviduct explants incubated in Hepes-TALP after 48 h, this medium was used in the remaining experiments.

Table 1: Effect of different media on sperm binding capacity after 30 min, 24 or 48 h of culture (geometric mean and 95% Confidence interval of the number of spermatozoa/ 0.1 mm² of oviduct explant; three replicates, 10 observations/replicate)
Sperm preservation method did not affect the overall or the time-dependent binding capacity within the same medium (P ≥ 0.05)

Sperm preservation method	Medium	Culture		
		30 min	24 h	48 h
Fresh	Hepes-TALP[a*]	79 (38-165)	35 (16-73)	23 (11-47)
	IVF-TALP[a†]	90 (43-188)	52 (25-108)	2 (0-3)
	TCM-199 + 10% FCS[b*†]	16 (7-34)	5 (2-11)	0 (0-1)
	TCM-199[b*†]	9 (4-19)	3 (1-8)	0 (0-1)
Frozen-thawed	Hepes-TALP[a*]	59 (28-123)	29 (14-62)	6 (2-12)
	IVF-TALP[ab†]	150 (71-313)	8 (3-16)	0 (0-1)
	TCM-199 + 10% FCS[b*†]	15 (7-32)	5 (2-9)	0 (0-1)
	TCM-199[b*†]	9 (5-24)	3 (2-9)	0 (0-1)

[ab] The overall binding capacity of spermatozoa for media within each preservation method with no superscript letters in common differs significantly ($P < 0.05$)
[*†] The time-dependent binding capacity of spermatozoa for media within each preservation method with no symbols in common differs significantly ($P < 0.05$)

Effect of the size of oviduct epithelial explants on sperm binding

After 30 min of coincubation, significantly more spermatozoa (two-sample *t*-test, $P < 0.001$) were bound per 0.1 mm² of oviduct epithelium when the surface area of the oviduct explants was < 20 000 µm² than when it was > 100,000 µm² (528 ± 70 and 85 ± 20, respectively). The coefficient of variation (CV) within the group of small oviduct explants (CV = 0.13) was smaller than in the group of large oviduct explants (CV = 0.24). As a result, only oviduct explants with a surface area < 20 000 µm² were used to investigate the relationship between NRR and sperm binding capacity.

Experiment 2: Relationship between NRR and capacity of spermatozoa to bind to oviduct epithelial explants

The relation between the number of spermatozoa bound to 0.1 mm² of oviductal epithelium and the NRR was dependent on time (Figure 3). The number of bound spermatozoa decreased significantly over time ($P < 0.001$).

The association between sperm binding capacity and NRR was also dependent on membrane integrity of the spermatozoa. At the start of coincubation, the initial mean percentage of membrane-intact spermatozoa in the tested sperm samples measured with SYBR®-14 and PI after thawing and washing of the spermatozoa ranged from 34 to 79% (Table 2).

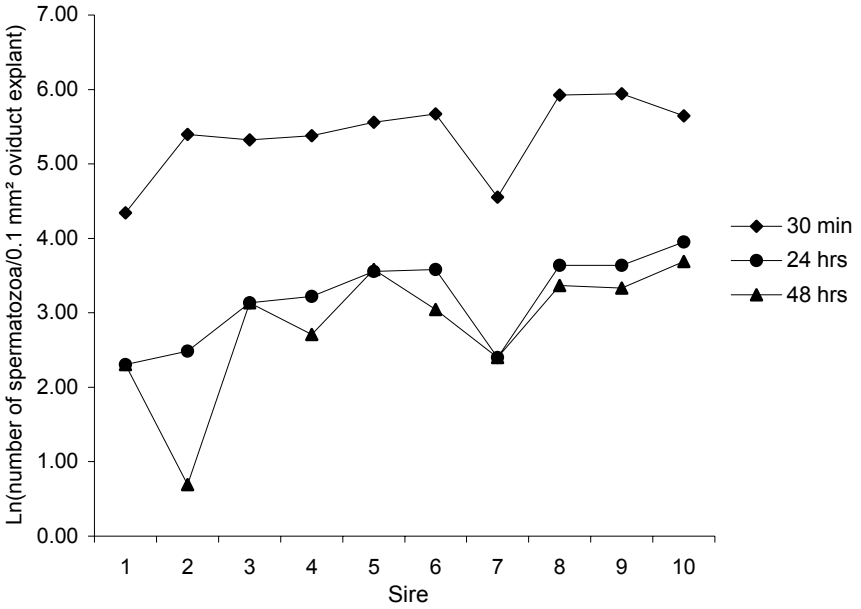

Figure 3: Binding capacity of spermatozoa of each bull to 0.1 mm² oviduct epithelium after 30 min, 24 and 48 h of culture (geometric mean of three replicates). Bulls are ranked according to increasing NRR. NRR and membrane integrity rates per bull are presented in Table 2

Table 2: Percentage of membrane-intact spermatozoa in the tested sperm samples as determined by SYBR-14® and PI staining after thawing and washing of the spermatozoa (Three replicates, mean ± SEM). Sires are ranked according to increasing NRR

		Membrane integrity (%)				
Sire	NRR	Replicate 1	Replicate 2	Replicate 3	Mean	SEM
1	52.8	44	45	39	43	2
2	54.0	52	56	46	51	3
3	58.6	73	68	73	71	2
4	64.7	54	48	46	49	2
5	64.9	75	85	78	79	3
6	65.9	80	77	71	76	3
7	66.7	32	32	38	34	2
8	66.9	68	61	63	64	2
9	68.4	71	65	67	68	2
10	69.9	85	77	75	79	3

A positive association between $\log_e$ transformed number of spermatozoa bound to 0.1 mm² of oviduct epithelium and the NRR was found after 24 h of co-incubation and only when the membrane integrity of the initial sperm sample was > 60% ($P< 0.05$; Table 3). Figure 4 shows the inverse predicted NRR and 95% confidence interval (CI) of a bull with membrane integrity of 85%. For example, when a mean of 40 spermatozoa were bound to 0.1 mm² of oviduct epithelium, the inverse predicted NRR of the bull is approximately 68% (95% CI: 61% to >70%).

Table 3: Regression lines* of the number of spermatozoa bound to epithelial oviduct explants versus the NRR and different membrane integrity (MI) rates after 24 h of culture

Membrane integrity (%)	Intercept ± se	Slope ± se	*P*
40	4.09 ± 2.08	- 0.03 ± 0.03	0.42
45	2.91 ± 1.81	- 0.01 ± 0.03	0.79
50	1.74 ± 1.64	0.01 ± 0.03	0.66
55	0.57 ± 1.60	0.03 ± 0.03	0.22
60	- 0.60 ± 1.71	0.05 ± 0.03	0.06
65	- 1.78 ± 1.94	0.07 ± 0.03	0.02
70	- 2.95 ± 2.26	0.09 ± 0.03	0.01
75	- 4.13 ± 2.63	0.11 ± 0.04	0.01
80	- 5.30 ± 3.03	0.13 ± 0.05	0.01
85	- 6.48 ± 3.45	0.15 ± 0.05	0.01

*For a given MI (%), the corresponding regression equation is: ln(no. spermatozoa) = a + b (NRR), were NRR = non-return rate (%), a = intercept and b = slope, e.g., if MI = 65%, then ln(no spermatozoa) = -1.78 + 0.07NRR

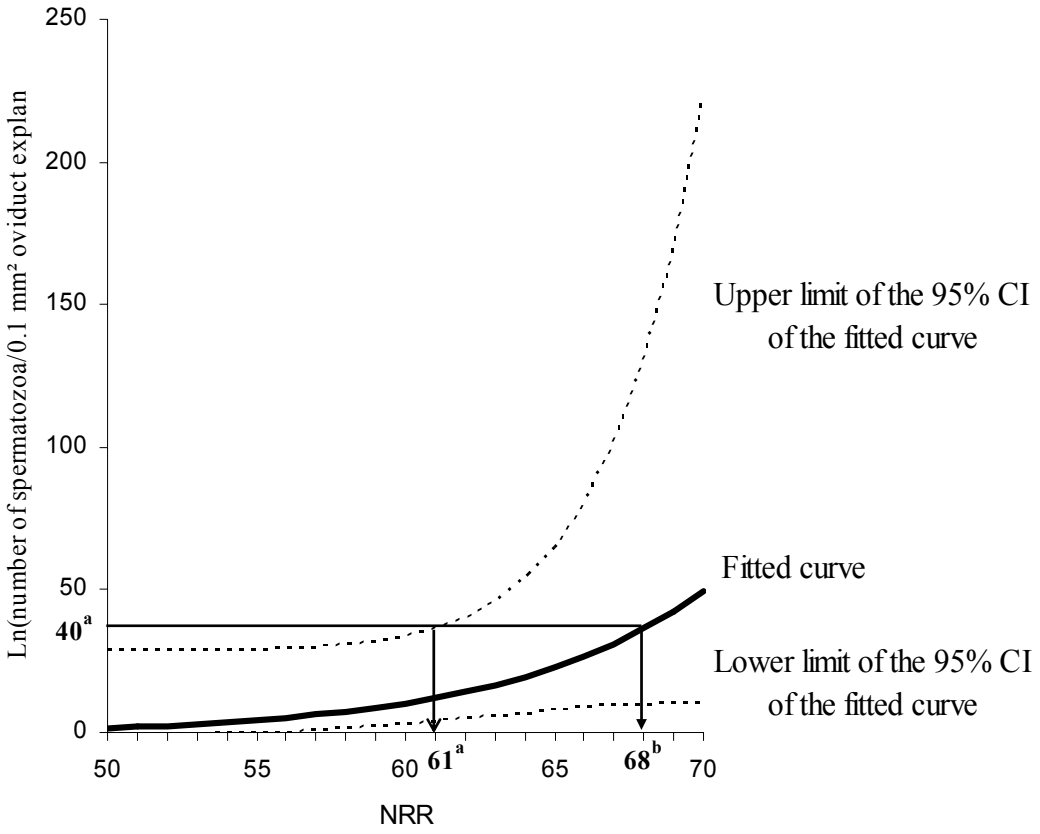

Figure 4: Inverse prediction of the NRR (95% CI) of a sperm sample with a membrane integrity of 85% and the given number of spermatozoa bound to the epithelial oviduct explant.

40^a: Number of spermatozoa/0.1 mm² of oviduct explant
68^b: Inverse predicted point estimate of the NRR given the number of spermatozoa/0.1 mm² of explant. The inverse predicted point estimate is obtained by using the intersection of the number spermatozoa/0.1 mm² with the fitted curve
61c: Inverse predicted lower 95% prediction limit. The inverse predicted lower 95% prediction limit is obtained by using the intersection of the number of spermatozoa/0.1 mm² of explant with the upper limit of the 95% CI of the fitted curve

Discussion

To study the binding of spermatozoa to oviduct epithelial explants, it is important to choose the right incubation medium in which membranes of both oviduct cells and spermatozoa remain intact for a minimum of 48 h. This aspect was very important in our study, in contrast to other studies in which only short incubation periods were used (Lefebvre et al., 1995, 1997; Petrunkina et al., 2001). In preliminary experiments, Hepes-TALP was considered as a more suitable medium for the survival of both spermatozoa and oviduct explants than were the other tested media: IVF-TALP, TCM-199 + 10% FCS, and TCM-199 alone. After 24 and 48 h of incubation, significantly more membrane-damaged

and capacitated spermatozoa were observed in the other media than in Hepes-TALP ($P < 0.05$; data not shown). Furthermore, more spermatozoa survived while bound to oviduct explants in Hepes-TALP in the presence of oviduct epithelial cells. All tested media except Hepes-TALP were bicarbonate buffered: it is known that bicarbonate induces capacitation of spermatozoa (Yanagimachi, 1994; Harrison et al., 1996). Capacitation appears to be involved in the release of bovine spermatozoa from oviduct epithelium (Lefebvre and Suarez, 1996) and in combination with decreased sperm survival is responsible for the important drop in sperm binding to oviduct explants after 24 h of coincubation in IVF-TALP. TCM-199 with or without the addition of 10% FCS was useful only for the incubation of oviduct explants but not for incubation of spermatozoa (unpublished data). Only a few spermatozoa survived in TCM-199, which is why binding capacity was lower in this medium. One could argue that dead spermatozoa could exert a negative effect on binding of remaining living spermatozoa to oviduct explants by the release of enzymes or other substances (Lefebvre and Suarez, 1996). However, this effect was not substantiated by a change in pH in any of the media. The tendency of frozen-thawed spermatozoa to have a lower binding capacity and shorter survival than fresh spermatozoa may be due to cell damage and the induction of capacitation-like changes during freezing and thawing procedures (Cormier et al., 1997). Moreover, capacitation is known to destabilize the sperm plasma membrane (Langlais and Roberts, 1985; Yanagimachi, 1994), and thus reduce the lifespan of spermatozoa (Watson, 1995).

Sperm binding should be investigated with oviduct explants smaller than 20 000 μm^2 to provide more repeatable results. Spermatozoa are not evenly distributed over the surface. They are spaced closely in some areas, and sparsely in others and are absent in a few areas, as has been observed previously (Lefebvre and Suarez, 1996). Sperm heads bind preferentially to cilia or in deeper regions of ciliated epithelial cells between cilia and not to secretory epithelial cells (Pollard et al., 1991; Petrunkina et al., 2001). Further experiments using electron microscopy are needed to determine whether oviduct explants with a small surface area consist of more ciliated epithelial cells and less secretory cells than do oviduct explants with a large surface area.

To investigate the binding density of bovine spermatozoa to oviduct epithelial explants, two very important criteria have to be met: sperm counting must be performed easily and repeatedly, and sperm binding must mimic the in vivo situation as closely as possible. The use of fluorescent dyes for the evaluation of sperm binding has two advantages. First, staining with JC-1, which labels the mitochondria of both spermatozoa

and oviduct cells, makes it easy to count the tails of spermatozoa bound to oviduct explants and to measure the surface area of the oviduct explant. Second, PI stains only membrane-damaged spermatozoa. In this way membrane-intact and membrane-damaged spermatozoa can be distinguished so that only membrane-intact spermatozoa are counted, which is not possible with videomicroscopy. Several methods for studying binding of spermatozoa to oviduct epithelial cells in mammals have been described (Thomas and Ball, 1996; Gualtieri and Talevi, 2000). Sperm binding density is usually evaluated on oviduct explants (Raychoudhury and Suarez, 1991; Thomas et al., 1994a; Lefebvre et al., 1995, 1997; Lefebvre and Suarez, 1996) or on oviduct monolayers (Pollard et al., 1991; Thomas et al., 1994b; Thomas and Ball, 1996; Ellington et al., 1999a; Gualtieri and Talevi, 2000). In our study, oviduct explants were used as a model for oviduct epithelial cells in vivo because of the maintenance of most of their morphological characteristics (Lefebvre et al., 1995). Because morphological differentiation and polarization of epithelial cells is more pronounced in polarized explants, there is higher sperm binding density in explants than in epithelial culture monolayers, as shown in humans (Baillie et al., 1997). The number of spermatozoa bound to living or fixed cocultures has been counted by means of scanning electron microscopy analysis (Pollard et al., 1991; Gualtieri and Talevi, 2000), videomicroscopy and image analysis (Lefebvre and Suarez, 1996; Lefebvre et al., 1995, 1997) or by labeling spermatozoa with the fluorochrome Hoechst 33342 followed by counting attached spermatozoa by means of image processing and analysis of fluorescent video images (Thomas et al., 1994a). Thomas at al. (1994a) were the first to develop a cytofluorescent assay for counting large numbers of labeled spermatozoa attached to somatic cell monolayers. However, the application of Hoechst 33342 leads to nuclear staining of both spermatozoa and oviduct explant cells, which makes it more difficult to distinguish spermatozoa from oviduct cells. This problem does not occur with JC-1. Although sperm heads and mitochondria in the oviduct cells also stain faintly positive, this staining does not interfere with the counting of spermatozoa.

Linking in vivo fertility with a relatively fast and cheap in vitro evaluation method would be very valuable for the prediction of fertility of a sire. The standard procedure for evaluating the fertility of semen from sires is to determine pregnancy data following AI. This procedure is time consuming and expensive because of the large number of young bulls entering the breeding program (Zhang et al., 1999). Several studies have already been done to find a simple and reliable test for fertility (Larsson and Rodriguez-Martinez, 2000). Despite the fact that a number of studies have focused on single sperm traits such as sperm

morphology (Barth, 1993), sperm motility (Stalhammar et al., 1994; Holt et al., 1997), and the presence of intact acrosomes (Cumming, 1995), none of these traits were correlated significantly with in vivo fertility. Because fertilization requires several sperm activities, it would be better to combine different sperm traits to achieve a better correlation between in vitro tests and in vivo fertility (Amann and Hammerstedt, 1993; Farrell et al., 1998; Zhang et al., 1999). With the in vitro model that we optimized, we established an association between density of sperm binding to oviduct explants and NRR. Spermatozoa that can bind to oviduct explants are characterized by an uncapacitated status (Lefebvre and Suarez, 1996), an intact acrosome (Gualtieri and Talevi, 2000), a superior morphology (Thomas et al., 1994b), and a normal chromatin structure (Ellington et al., 1999b). Thundathil et al. (1999) demonstrated that the proportion of uncapacitated spermatozoa present in frozen-thawed bull semen varies among bulls and, more important, that the presence of uncapacitated spermatozoa is positively correlated with fertility. Uncapacitated spermatozoa have an advantage over capacitated spermatozoa during their transit to the site of fertilization in the oviduct because they are more likely to survive. If capacitation were to occur before spermatozoa reached the oviduct, the sperm population available for fertilization may be reduced, causing an adverse effect on fertilization. When the percentage of uncapacitated spermatozoa in a sperm sample is high, more spermatozoa are able to bind to oviduct explants, which may result in a higher fertility rate. Differences among individual animals in the capacity of spermatozoa to bind to oviduct explants in vitro have already been reported for stallions and boars (Thomas and Ball, 1996; Petrunkina et al., 2001). However in these studies no data were available on the comparative ranking of fertility, so the relationship between sperm-oviduct binding index and fertility could not be established.

Using our in vitro model, we found that the number of spermatozoa bound to oviduct explants coincubated for 24 h is positively associated with in vivo bull fertility only when the membrane integrity of the initial sperm sample > 60%. No association between the number of spermatozoa bound to oviduct explants and NRR was found after 30 min and 48 h of coincubation and after 24 h of coincubation when the membrane integrity of the sperm sample was < 60%.

The model can be used for predicting NRR of bulls when sperm samples with membrane integrity rates > 60% are coincubated with oviduct explants for 24 h. However, the width of the 95% CI is large and could be narrowed by collecting more data to predict

NRR and by controlling for factors that can bias NRR, such as technician and age and parity of the cow. Moreover, testing of multiple ejaculates per bull could also increase the accuracy of the model.

In the present study, labeling of both spermatozoa and oviduct explants with the fluorescent carbocyanine dye JC-1 in combination with PI without fixing provided a rapid, reliable, and reproducible method for counting the number of membrane-intact spermatozoa bound to living oviduct explants. The results of this analysis indicate that the capacity of spermatozoa bound to oviduct explants in vitro varies among bulls and that the number of spermatozoa bound to oviduct epithelial explants is positively associated with the NRR when evaluated after 24 h of sperm-oviduct incubation provided that the membrane integrity of the initial sperm sample is > 60%.

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SPERM-OVIDUCT BINDING CAPACITY OF COOLED BOVINE SPERMATOZOA STORED FOR SEVERAL DAYS IN DIFFERENT DILUENTS

Manuscript in preparation

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Abstract

In this in vitro study, a previously developed sperm-oviduct binding assay is used to analyze oviduct binding capacity of spermatozoa after storage in three different diluents (CAPROGEN®, Triladyl® and CEP). Bovine semen was collected by means of an artificial vagina connected with a tube containing 5 ml Triladyl®-diluent. Both diluent and seminal plasma were removed by centrifugation (720 x g, 10 min). The sperm concentration was adjusted to 10 x 10^6 spermatozoa/ml. Spermatozoa were used immediately (D_0-spermatozoa) or after storage in the three diluents at 4°C for up to 2, 4 and 6 days (D_2-, D_4- and D_6-spermatozoa). This experiment was performed using oviduct explants composed of membrane-intact oviduct cells with high mitochondrial membrane potential and with beating cilia. No significant effect of diluent on sperm-oviduct binding capacity of D_2-, D_4- and D_6-spermatozoa was observed after 30 min, 24 and 48 h of co-culture. Sperm-oviduct binding capacity of D_0- spermatozoa was different over time from the binding capacity of D_2-, D_4- and D_6-spermatozoa previously stored in the three diluents ($P < 0.001$). In this respect, the number of D_2-, D_4- and D_6-spermatozoa attached to oviduct explants decreased significantly ($P < 0.001$; Figure 3) during the time of co-culture. In contrast, more than 90% of D_0-spermatozoa attached to oviduct explants were released before 24 h of co-culture and 15% of these progressively motile released spermatozoa reattached to oviduct explants between 24 and 48 h of co-culture.

In an additional experiment, it was shown that oocyte penetration rate of spermatozoa stored at 4°C for up to 6 days was also not significantly affected by diluent type.

Introduction

Fertilization involves a highly coordinated sequence of cellular interactions between male and female gametes. To maximize fertilization success, it is important that an appropriate number of spermatozoa meets the oocyte under proper physiological fertilization conditions at the right time and at the right place (Töpfer-Petersen, 1999). In order to obtain this optimal event, spermatozoa are selected quantitatively and qualitatively during their transit in the female reproductive tract (Jouannet and Feneux, 1987). This means that only morphologically normal and progressively motile spermatozoa are able to migrate through the cervical mucus and reach the oviduct (Jeulin et al., 1985), where they are stored until ovulation (Hunter, 1996; Suarez, 1998).

Recently, an in vitro bio-assay to quantify the number of 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1)-labeled spermatozoa bound to a defined oviduct explant surface area was developed in our laboratory (De Pauw et al., 2002). It was shown that this in vitro system provides a rapid, reliable and reproducible method for counting the number of spermatozoa bound to oviduct explants. By means of this assay, a clear difference in sperm binding capacity among individual bulls was observed and evidence was obtained that sperm binding ability to oviduct explants is related to in vivo fertility of the sperm donor.

The first part of the present study was focused on further refinement of this new in vitro model. The aim of the second part was to use this sperm-oviduct binding assay as a functional test to examine sperm quality after storage in different sperm diluents for several days. The effect of different oviduct explant cultures on the binding capacity of 4-days stored spermatozoa in Cauda Epididymal Plasma (CEP)-diluent at 5°C was investigated first in order to increase the accuracy of the assay. This system was then used in the second experiment to compare oviduct binding capacity of spermatozoa stored in three different diluents (CEP, CAPROGEN® and Triladyl®) for several days (0, 2, 4 and 6 days). CAPROGEN® is a commercially available diluent extensively used for liquid storage of bovine semen at ambient temperature (Shannon, 1965; Vishwanath and Shannon, 2000). Triladyl® is a TRIS-based diluent commonly used for frozen semen. Good results have also been obtained with liquid semen stored at room temperature (RT) or at 4°C (De Pauw et al., 2003a). The Cauda Epididymal Plasma (CEP)-diluent, was recently developed in our laboratory for storage of bovine spermatozoa at 4°C (Verberckmoes et al, in preparation). In the final experiment, in vitro oocyte penetration rate of spermatozoa

previously stored in the same three diluents at 4°C for up to 6 days was examined in order to get extra information on the quality of spermatozoa stored in the different diluents.

Materials and Methods

Chemicals and media

Unless otherwise stated, chemicals and media were obtained from Sigma (Bornem, Belgium).

Media

Hepes buffered TALP medium contained 114 mM NaCl, 3.1 mM KCl, 0.3 mM NaH_2PO_4, 2.1 mM $CaCl_2$, 0.4 mM $MgCl_2$, 2 mM $NaHCO_3$, 0.2 mM sodium pyruvate, 10 mM sodium lactate, 10 µg/ml gentamycin sulphate, 10 mM Hepes and 3 mg /ml bovine serum albumin.

Modified bicarbonate buffered TCM-199 (Gibco BRL Life Technologies, Merelbeke, Belgium) supplemented with 0.2 mM sodium pyruvate, 0.4 mM glutamine, 50µg/ml gentamycin sulphate, 2.5 µg/ml fungizone (Gibco BRL Life Technologies, Merelbeke, Belgium) and 10% fetal calf serum was used for overnight culture of oviduct cells.

Maturation medium consisted of modified bicarbonate buffered TCM-199 medium (Gibco BRL Life Technologies, Merelbeke, Belgium) supplemented with 20% heat-inactivated foetal calf serum (FCS) (N.V. HyClone Europe S.A., Erembodegem, Belgium), 0.2 mM sodium pyruvate, 50 µg/ml gentamycin sulphate and 0.4 mM glutamine.

Fertilization medium consisted of Tyrode's solution without Hepes supplemented with 25 mM $NaHCO_3$, 0.2 mM sodium pyruvate, 10 mM sodium lactate, 10 µg/ml gentamycin sulphate, 6 mg/ml fatty acid-free BSA and 10 µg/ml heparin.

Media were passed through sterile 0.22 µm Acrodisc® Syringe low protein binding filters (Millipore Corporation, New Bedford, MA) before being used.

Sperm diluents

Three diluents were used in the present study:

Triladyl® (Minitüb, Tiefenbach b. Landshut, Germany) a TRIS-based diluent is prepared by adding 20% egg yolk to the Triladyl concentrate containing 6.7% glycerol.

CAPROGEN® (Livestock Improvement Corporation, Hamilton, New Zealand) commercial diluent containing caproic acid (0.025g/100 ml) and 5% egg yolk. The diluent was purged with nitrogen to reduce its concentration of oxygen (Krzyzosiak, 2000).

CEP a simple sperm diluent based on the composition of bovine cauda epididymal plasma (Verberckmoes et al., in preparation) containing 10% egg yolk.

Sperm preparation

Ejaculates were collected from a 3-year-old Red Pied bull by means of an artificial vagina connected with a tube containing 5 ml Triladyl®-diluent. Immediately after collection, spermatozoa were separated from the seminal plasma and diluent by centrifugation (720 x g, 10 min) at RT. The progressive motility was subjectively assessed by visual estimation under a light microscope (Leica DMR, Van Hopplynus NV, Brussels, Belgium) (magnification x 200) equipped with a stage warmer (37°C). The nucleic stains SYBR® 14 and propidium iodide (PI) (LIVE/DEAD® Sperm Viability Kit (Molecular Probes, Leiden, The Netherlands)) were used for analyzing membrane integrity of spermatozoa by means of fluorescence microscopy. Sperm concentration was determined using a Bürker chamber and then adjusted to a concentration of 10 x 10^6 spermatozoa/ml diluent. After storage at 4°C, spermatozoa were washed with Hepes-TALP by centrifugation (720 x g, 10 min). Sperm concentration of the pellet was determined and subsequently diluted to a concentration of 1 x 10^6 spermatozoa/ml Hepes-TALP.

Collection and processing of oviducts for culture

Oviducts were obtained from local abattoirs and transported immediately to the laboratory in ice-cold physiological saline (0.9% NaCl). After rinsing in Hepes-TALP, oviducts were carefully dissected free of the surrounding tissue and rinsed again in Hepes-

TALP. The lumen was cut open longitudinally with sterile scissors and lightly scraped with a sterile scalpel to collect epithelial tissue. Sheets of epithelial cells were washed three times with 5 ml Hepes-TALP by sedimentation followed by supernatant removal. The last washing was performed in modified TCM-199 medium + 10% FCS. After supernatant removal, the cell sheets were disaggregated into smaller pieces by passing twice through a 26-gauge needle connected to a 1-ml syringe.

Two hundred microlitres of sedimentated oviduct explants were added to 5 ml modified TCM-199 medium + 10% FCS and cultured overnight in a 50-ml flask at 38.5°C in 5% CO_2 in air. After overnight culture, the oviduct cells had formed clumps or worms with beating cilia. The medium containing oviduct explants was transferred to a 15-ml tube and the supernatant was removed after sedimentation. Two hundred oviduct explants with a surface smaller than 20.000 µm² were co-cultured with 1 x 10^6 spermatozoa/ml Hepes-TALP at 38.5°C in air in a four-well culture plate.

Quantification of sperm binding to oviduct explants

After co-culture, minimum 10 oviduct explants were rinsed in Hepes-TALP to remove loosely attached spermatozoa, and transferred to fresh Hepes-TALP. The oviduct explants were stained with JC-1 (Molecular Probes, Leiden, The Netherlands) to label mitochondria of both spermatozoa and oviduct explants in order to count the number of bound spermatozoa, and with PI to identify membrane-damaged spermatozoa. Density of bound spermatozoa on one side of the oviduct explant was determined by means of fluorescence microscopy performed by using a Leica DMR microscope (Van Hopplynus N.V., Brussels, Belgium) equipped with an excitation filter of 450 - 490 nm from a 100 W mercury lamp and examined at a magnification 400 x. The surface of the oviduct explant was measured with the Image Database Program of Leica (Van Hopplynus NV, Brussels, Belgium). The number of spermatozoa bound to 0.1 mm² of oviduct explant surface was used as the parameter of binding capacity.

In vitro oocyte maturation and fertilization

Bovine ovaries collected from a local abattoir were transported to the laboratory within 2 hours. Follicles of 2 to 6 mm in diameter were gently aspirated and cumulus-oocyte complexes (COC) were matured in 500 µl maturation medium at 38.5 °C in 5%

CO_2 in air at 100% humidity for 24-26 hours. After maturation, 50 COC were incubated with a final sperm concentration of 1 x 10^6 spermatozoa/ml for 20 h.

For the assessment of oocyte penetration rate, the presumed zygotes were vortexed in 2 ml Hepes-TALP for 2 min to remove excess spermatozoa and cumulus cells, and fixed in 2% glutaraldehyde and 2% paraformaldehyde in phosphate buffered saline for at least 24 h. After fixing, zygotes were stained with 10 µg/ml Hoechst 33342 (Molecular Probes, Leiden, The Netherlands) for 10 min and evaluated with a Leica DMR fluorescence microscope for signs of oocyte penetration. Successful oocyte penetration was characterized by the presence of maternal and paternal pronuclei (fertilization rate). Oocytes with more than two pronuclei (polyspermy rate) were also considered penetrated.

Experimental design

Experiment 1: Effect of oviduct explant cultures on the binding capacity of spermatozoa stored in CEP-diluent at 4°C for up to 4 days

This experiment was performed with 5 oviduct explant cultures derived from 5 different oviducts. Spermatozoa were collected and processed as described above. Spermatozoa were stored at a concentration of 10 x 10^6 spermatozoa/ml CEP-diluent at 4°C in darkness for up to 4 days. After storage, spermatozoa were washed with Hepes-TALP by centrifugation (720 x g, 10 min). Two hundred oviduct explants with a surface smaller than 20 000 µm² were co-cultured with 1 x 10^6 spermatozoa/ml Hepes-TALP at 38.5°C in air in a four-well culture plate. After 30 min of co-culture, the number of spermatozoa bound to 10 oviduct explants were counted and expressed as the number of spermatozoa bound to 0.1 mm² oviduct explant.

Experiment 2: Effect of diluent and duration of storage on the binding capacity of spermatozoa

Every other day, ejaculates were collected in 5 ml Triladyl®-diluent to obtain spermatozoa of different ages. Spermatozoa were used immediately (D_0- spermatozoa) or after storage in 3 different diluents (CEP, CAPROGEN® and Triladyl®) at a concentration of 10 x 10^6 sp/ml at 4°C in darkness for 2 (D_2-spermatozoa), 4 (D_4-spermatozoa) and 6 (D_6-spermatozoa) days. After storage, spermatozoa were washed with Hepes-TALP by centrifugation (720 x g, 10 min). Membrane integrity (SYBR®-14/PI) and progressive

motility (subjectively) of all sperm samples were determined. Spermatozoa were then co-cultured with two hundred oviduct explants with a surface smaller than 20 000 µm², derived from the same oviduct explant batch, at 38.5°C in air in a four-well culture plate. After 30 min, 24 and 48 h of co-culture, at least 10 oviduct explants were stained with JC-1 and PI for 15 min. The number of spermatozoa bound to 10 oviduct explants were counted and expressed as the number of spermatozoa bound to 0.1 mm² oviduct explant. This experiment was repeated three times.

Experiment 3: In vitro oocyte penetration rate of spermatozoa stored in different diluents at 4°C for up to 6 days

Spermatozoa were collected in 5 ml Triladyl®-diluent and processed as described above. After storage in CEP, CAPROGEN® and Triladyl® at 4°C in darkness for 6 days, spermatozoa were washed with Hepes-TALP by centrifugation at RT (720 x g, 10 min) and the supernatant was removed. Motile and membrane-intact spermatozoa from the pellet were separated on a discontinuous Percoll®-gradient. The sperm concentration was adjusted with fertilization medium to obtain a final sperm concentration of 1 x 10^6 spermatozoa/ml. Cumulus oocyte complexes (n = 946, 3 replicates) were co-cultured with spermatozoa, previously stored in the three diluents at 4°C for up to 6 days, for 20 h. Finally, presumed zygotes were vortexed, fixed, stained with Hoechst 33342 and examined with a Leica DMR fluorescence microscope for signs of penetration. This experiment was repeated three times.

Statistical analysis

Difference in mean number of spermatozoa attached to 10 oviduct explants from 5 different cultures was examined using analysis of variance (ANOVA) methods through Generalized Linear Models (SPSS 10.0, Chicago, USA). Differences were considered significant at $P = 0.05$.

Difference in membrane integrity and progressive motility of D_0-, D_2-, D_4- and D_6-spermatozoa stored in CEP, CAPROGEN® and Triladyl® at 4°C was examined using analysis of variance (ANOVA) methods through Generalized Linear Models (SPSS 10.0, Chicago, USA). Differences were considered significant at $P = 0.05$.

The effect of diluent and duration of storage on sperm binding capacity to oviduct explants was analyzed with the Mixed procedure of SAS V8 (SAS Institute Inc.). The

outcome variable was expressed as the $\log_e$ transformed number of spermatozoa bound per 0.1 mm² oviduct explant. Explanatory variables were diluent and duration of storage. Both explanatory variables were included as class variables. The co-culture time was considered a repeated measure and was evaluated as class variable. Replicate was included as random variable to take into account the variation between the replicates.

Difference in oocyte penetrating ability of spermatozoa stored in CEP, CAPROGEN® and Triladyl® at 4°C for up to 6 days, and frozen-thawed control spermatozoa was examined using analysis of variance (ANOVA) methods through Generalized Linear Models (SPSS 10.0, Chicago, USA). Differences were considered significant at $P = 0.05$.

Results

Experiment 1: Effect of oviduct explant cultures on the binding capacity of spermatozoa stored in CEP-diluent at 4°C for up to 4 days

When the binding capacity of CEP-stored spermatozoa to oviduct explants obtained from different oviducts cultured under the same in vitro conditions was compared, no significant differences (Figure 1) were observed. The mean (± SEM) binding capacity of spermatozoa attached to different oviduct cultures was 562 ± 140, 130 ± 13 and 71 ± 12 spermatozoa/0.1 mm² after respectively 30 min, 24 h and 48 h of co-culture.

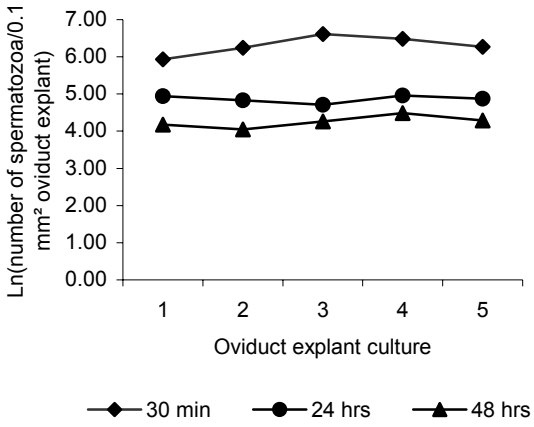

Figure 1: Binding capacity of spermatozoa stored in CEP-diluent for up to 4 days to 5 different oviduct explant cultures evaluated after 30 min, 24 and 48 h of co-culture. (Geometric mean of three replicates; 10 observations/replicate)

Experiment 2: Effect of diluent and duration of storage on the binding capacity of spermatozoa

No significant diluent effect on progressive motility and membrane integrity of D_2-, D_4- and D_6-spermatozoa was observed. These percentages were significantly ($P < 0.05$) lower than the percentages of progressive motility and membrane integrity of D_0-spermatozoa (Figure 2). Attachment of D_0-, D_2-, D_4- and D_6-spermatozoa to oviduct explants occurred within 30 min of co-culture. Initially, most, if not all, of the attached spermatozoa showed vigorous flagellar activity when evaluated microscopically. This flagellar motion declined over time in co-culture. Statistical analysis of the data showed no significant difference in the binding capacity for D_2-, D_4- and D_6-spermatozoa stored in the different diluents when measured 30 min, 24 and 48 h after start of co-culture (Figure 3). At 30 min and 48 h of co-culture, a significantly higher number of D_0-spermatozoa was bound to oviduct explants than of D_4- and D_6-spermatozoa ($P < 0.001$; Figure 3), but no significant difference was found after 24 h of co-culture.

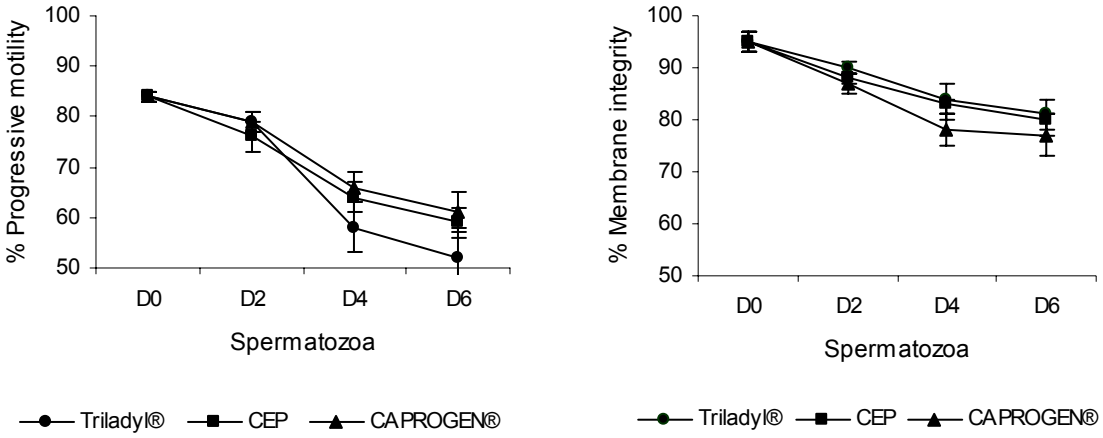

Figure 2: Percentage of progressive motility and membrane integrity of D_0-, D_2-, D_4- and D_6-spermatozoa stored in Triladyl®, CAPROGEN® or CEP at 4°C, evaluated before co-culture with oviduct explants. (Mean ± SEM of three replicates)

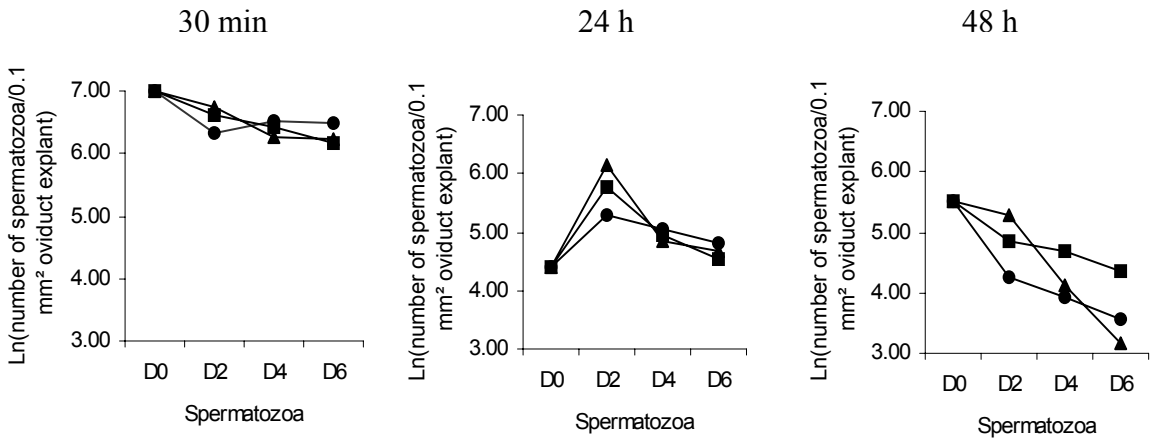

Figure 3: Binding capacity of spermatozoa stored for 0, 2, 4 and 6 days in Triladyl® (●), CAPROGEN® (▲) and CEP (■) to oviduct explants evaluated after 30 min, 24 and 48 h of co-culture (Geometric mean of three replicates; 10 observations/replicate)

Interestingly, the overall binding capacity of D_0-spermatozoa is different over time than the overall binding capacity of D_2-, D_4- and D_6-spermatozoa stored in the different diluents ($P < 0.001$; Figure 4). The number of D_2-, D_4- and D_6-spermatozoa attached to oviduct explants decreased significantly ($P < 0.001$; Figure 3) during the time of co-culture. While more than 90% of D_0-spermatozoa attached to oviduct explants were released before 24 h of co-culture and 15% of these progressively motile released spermatozoa reattached to oviduct explants between 24 and 48 h of co-culture.

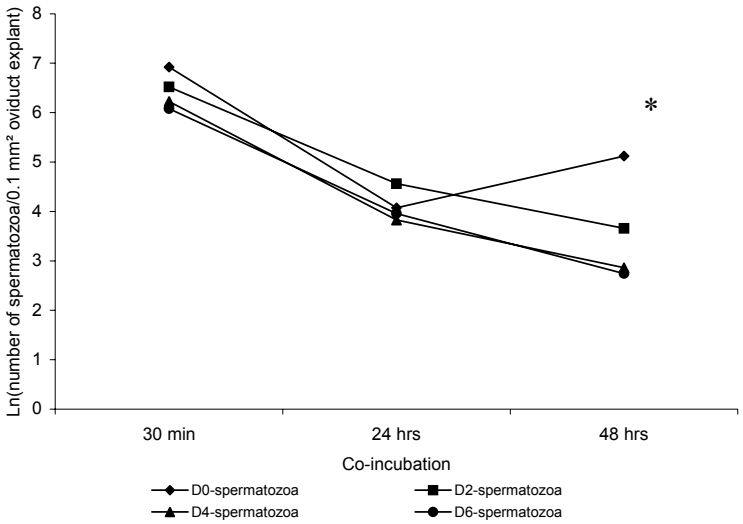

Figure 4: The overall binding capacity of spermatozoa stored for 0, 2, 4 and 6 days in the different diluents to oviduct explants measured after 30 min, 24 and 48 h of co-culture (Geometric mean of three replicates; 10 observations/replicate)

*The binding capacity of D_0-spermatozoa is different over time of co-culture than the binding capacity of D_2-, D_4- and D_6-spermatozoa

Experiment 3: In vitro oocyte penetration rate of spermatozoa stored in different diluents at 4°C for up to 6 days

Oocyte penetration rate did not significantly differ for spermatozoa previously stored in CAPROGEN® (61%) and CEP (67%) at 4°C for up to 6 days (Figure 5). These percentages were also not significantly different from the percentage of oocytes penetrated by frozen-thawed spermatozoa, used as control in our IVF-experiments (71%). The oocyte penetration rate of spermatozoa stored in Triladyl® could not be measured because of repetitive diluent contamination.

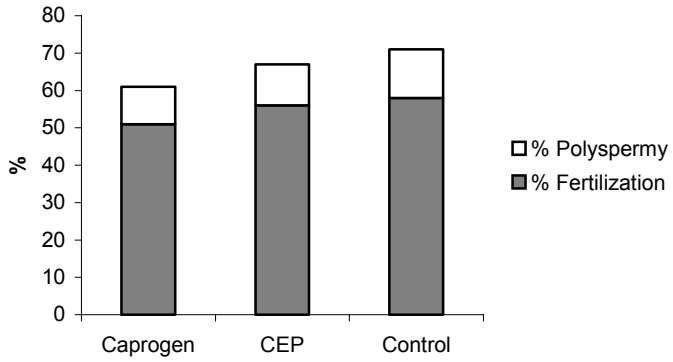

Figure 5: Fertilization and polyspermy rates of spermatozoa stored in CAPROGEN® or CEP at 4°C for up to 6 days, and of frozen spermatozoa of a control bull used immediately after thawing

Discussion

In order to apply our newly developed sperm-oviduct assay (De Pauw et al., 2002) to evaluate binding capacity of cooled bovine spermatozoa, the effect of explants prepared from different oviducts on the binding capacity of spermatozoa was examined. The results of this experiment indicated no significant effect of oviduct explant culture on the binding capacity of cooled bovine spermatozoa previously stored in CEP-diluent for up to 4 days. This could partially be explained by the fact that attachment of spermatozoa to oviduct explants is independent on the cycle stage of the cow from which oviduct explants were recovered (Lefebvre et al., 1995). However, other factors concerning quality of in vitro prepared oviduct explants may also exert an effect on sperm binding capacity and that is why in our experiments oviduct explants must fulfil additional criteria. Firstly, only oviduct explants with an area surface smaller than 20 000 µm² were selected, which has been reported in an earlier study, to provide more repeatable results (De Pauw et al., 2002).

Secondly, oviduct explants must also be evaluated for viability, which was assessed in each experiment by evaluating their ciliary activity (subjectively), membrane-integrity (SYBR®-14/PI) and mitochondrial membrane potential (JC-1). Because it is known that spermatozoa will not bind to non-viable oviduct explants, only oviduct explants composed of membrane-intact cells with high mitochondrial membrane potential and with beating cilia were used in our experiments, which improves the accuracy of our sperm-oviduct binding assay.

Our sperm-oviduct binding assay was used to examine the effect of different diluents (CAPROGEN®, CEP and Triladyl®) on the oviduct binding capacity of spermatozoa stored in these different diluents for up to 0, 2, 4 and 6 days. The sperm-oviduct binding assay was used since it is widely believed that attachment of spermatozoa to oviduct explants in co-culture is a process that selects the higher quality spermatozoa from a given sample, including morphologically normal and motile spermatozoa with intact plasma and acrosomal membranes and higher quality chromatin structure (Thomas et al., 1994; Ellington et al., 1999). These sperm characteristics are of utmost importance, because damage to acrosome, plasma membrane and flagellum occurs independently (McLaughlin et al., 1993). Damage to sperm chromatin can occur or be present without a concomitant decrease in sperm motility (Twigg et al, 1998; Evenson et al., 1999) and hence be missed by conventional sperm analysis. To allow a direct comparison between different diluents, spermatozoa needed to be stored under the same conditions. In our study, spermatozoa were collected in Triladyl®-diluent during ejaculation (De Pauw et al., 2003b) before being stored in the different diluents at 4°C for up to 2, 4 and 6 days. Our data showed that all diluents have the same capacity to preserve a similar concentration of higher quality, noncapacitated spermatozoa bound to oviduct explants at each time point. In these cases, viable spermatozoa rapidly attached and remained bound to oviduct explants in substantial numbers for up to 48 h of co-culture. Furthermore, a similar tendency in spermatozoa release was observed during co-culture. Some of the attached spermatozoa became non-viable and were released as a consequence during the time of co-culture. Another proportion of released spermatozoa was still membrane-intact, as measured with SYBR®-14/PI, but became rapidly immotile. One possibility is that these spermatozoa were capacitated and underwent a short period of hyperactivated motility before becoming immotile (Talevi and Gualtieri, 2001). In the present experiment, the capacitation status of spermatozoa was not investigated with chlortetracyclin (CTC) staining because this method is not reliable in our hands. Indirect determination of

capacitation status was performed by means of Pisum Sativum agglutinin staining, which measures the acrosome reaction, the next step in the capacitation process. The fact that a higher proportion of released spermatozoa were acrosome-reacted and the fact that this proportion increased when spermatozoa were stored for a longer period of time (data not shown), may suggest that these spermatozoa were released due to changes in their capacitation status. Furthermore, these released spermatozoa were not able to reattach to oviduct explants anymore. In contrast to this observation, we found that sperm detachment and reattachment to oviduct explants occur readily when spermatozoa were added to oviduct explants during ejaculation without storage (D_0-spermatozoa). Such transient attachment and reattachment was also observed in human spermatozoa (Ellington et al., 1998). In our study, the weaker binding of these D_0-spermatozoa to oviduct explants is probably due to the collection of spermatozoa into egg yolk containing diluent (Triladyl®-diluent) immediately after ejaculation (De Pauw et al., 2003b). This sperm collection method limits the contact time between spermatozoa and seminal plasma within seconds. (De Pauw et al., 2003b). Manjunath et al. (2002) discovered that the low-density lipoproteins (LDL) of egg yolk especifically bind detrimental BSP proteins of seminal plasma. This binding is rapid, specific, saturable and stable even after ultracentrifugation of semen, and could avoid the binding of BSP proteins on the sperm membrane. Furthermore, LDL are believed to act at the level of sperm membrane and in this way it can also avoid or reduce the binding of BSP proteins on the sperm membrane (Vishwanath et al., 1992).

It has been shown that one of these BSP proteins, identified as PDC-109, associates with the sperm surface during ejaculation (Desnoyers and Manjunath, 1993). This seminal plasma protein is responsible for the binding of spermatozoa to fucosylated molecules on oviductal epithelium (Ignotz et al., 2001; Suarez et al., 2002). Its importance in the binding of spermatozoa to oviduct epithelium could also be illustrated by the fact that only 7% of epididymal spermatozoa, which are not associated with PDC-109, was able to attach to oviduct epithelial cells, whereas 75% of ejaculated spermatozoa from the same bull attached (Ellington et al., 1999). This is a very important observation since in our study, a high proportion of D_0-spermatozoa were rapidly detached without a significant drop in motility. A possible reason could be a decreased presence or complete absence of PDC-109 proteins on the surface of “coated” spermatozoa resulting in a weaker oviduct binding, which can easily be overcome by mechanical forces exerted by D_0-spermatozoa, exhibiting excellent progressive motility. The fact that BSP proteins bind to LDP of egg yolk could

suggest that this interaction could be responsible for the binding of egg yolk coated spermatozoa to oviduct explants. However, further experiments are needed to confirm this hypothesis. The significantly lower values of different motility characteristics of D_2-, D_4- and D_6-spermatozoa than of D_0-spermatozoa (Verberckmoes et al., in preparation) could probably be the reason why D_2-, D_4- and D_6-spermatozoa remain bound to oviduct explants and could only be released due to capacitation or when they became non-viable.

In the final experiment it was examined whether prolonged storage of spermatozoa in the different diluents affected oocyte penetration rates of D_6-spermatozoa. No significant difference in oocyte penetration rate was observed for D_6-spermatozoa stored in CEP (67%) and CAPROGEN® (61%). Unfortunately, the penetration rate of spermatozoa stored in Triladyl®-diluent could not be measured because of repetitive diluent contamination. However, it was earlier reported that "coated" spermatozoa stored in Triladyl® yield a penetration rate of 71% (De Pauw et al., 2003a).

In summary, the use of high quality oviduct explants further improves the accuracy of our sperm-oviduct binding assay and provides a rapid, reliable and reproducible method for counting the number of spermatozoa bound to oviduct explants in vitro. Using this sperm-oviduct binding assay, the three tested diluents yielded similar concentrations of high quality spermatozoa. This could be concluded from the fact that for the three diluents no significant difference was observed at the level of sperm-oviduct binding or oocyte penetration rate. Whether this sperm-oviduct binding assay can be used as an indicator for in vitro fertilizing capacity of a sperm sample needs to be investigated by using semen from several bulls with pronounced differences in in vitro fertilization.

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9.

GENERAL DISCUSSION

General discussion

During the 1950s and 1960s, prolonged storage of liquid bovine semen was the subject of many investigations (review: Vishwanath and Shannon, 2000). However, since the commercialization of CAPROGEN®, a diluent developed in the early 1980s (Shannon and Curson, 1984), no further research has been performed on this subject. Until now, many diluents have been designed, ranging from a simple salt solution to a more complex buffered medium supplemented with egg yolk, glycerol, antibiotics and energy substrates. Most of these diluents are used for the storage of spermatozoa at a low temperature (4°C) to reduce sperm metabolic activity and maintain sperm quality. Alternative methods of metabolic inhibition for stored spermatozoa are CO_2-gassing (Illini Variable Temperature (IVT-) diluent; Van Demark and Sharma, 1957) and N_2-gassing (CAPROGEN® diluent; Shannon and Curson, 1984). In field trials, both diluents have been proven to maintain the fertilizing ability of spermatozoa stored for three days at room temperature (RT).

To design a new diluent for sperm storage of up to several days, two important factors which influence the quality of spermatozoa after ejaculation and during in vitro storage must be taken into account: the composition of the storage medium and the temperature at which semen is stored after dilution.

Concerning the composition of the storage medium, we started with the idea that the luminal environment of the cauda epididymidis has the capacity to store densely packed bovine spermatozoa in a potentially fertilizing condition for several weeks prior to ejaculation (Cascieri et al., 1976). This phenomenon suggests that cauda epididymal plasma could be responsible for both reversible suppression of motility and prolonged viability observed in vivo. This encouraged us to hypothesize that cauda epididymal constituents may help in the formulation of a better diluent for preserving spermatozoa at RT or at 4°C **(chapter 2 and 4)**. Therefore, specific physicochemical conditions, ionic composition and protein secretions of bovine cauda epididymidis were investigated. In **chapter 4**, spermatozoa were stored in the simple salt solution Hepes-TALP at RT for up to 4 days. This medium was modified to obtain physicochemical conditions similar as in cauda epididymal plasma **(chapter 2)**. In our study, the pH of Hepes-TALP (pH 7) was decreased to pH 4, 5 or 6 by addition of 1 M HCl (strong acid). From the results it was clear that a decrease in pH below 6 was spermicidal and caused immediate cessation of sperm motility. When spermatozoa were stored at pH 6 or 7, which corresponds to in vivo values of cauda epididymal plasma and seminal plasma (pH 6.7), sperm motility was less

than 30% after 2 days and was lost after 3 days of storage. Sperm motility could no longer be reactivated and therefore, these spermatozoa were assumed nonviable. Jones and Bavister (2000) used the weak acid CO_2 in their experiments to lower the pH of the medium to 6.8. They found that spermatozoa remained completely immobilized and that sperm motility could be completely reactivated for a maximum period of 2 days. In conclusion, reversible immobilization of sperm motility by using a strong acid is not possible. CO_2-gassing, which is less practical, could be used if the spermatozoa were stored for less than 2 days.

Hyperosmotic storage conditions (compared to both seminal and blood plasma: 300 mOsm) used to store bovine spermatozoa (354 mOsm) were also ineffective for long-term preservation in vitro. In hibernating bats, sperm storage in the epididymis occurs at extreme osmolarities of about 1500 mOsm, which is 5 times as high as that of plasma. Hyperosmolarity leads to temporary dehydration and is a well-recognized mechanism by which certain organisms remain viable in times of environmental stress. However, by using the same osmolyte (sorbitol) as Crichton et al. (1994) used in their in vitro studies, we were not able to obtain a reduction of bull sperm motility or to maintain sperm quality. Until now, the osmolyte responsible for increasing osmolarity in hibernating bats has not been identified. According to Crichton et al. (1994), several osmolytes such as simple sugars and sugar alcohols (including glycerol), amino acids, urea and carnitine present in an effective concentration can already be eliminated. The improvement of sperm survival by dehydration still remains a very interesting approach. In his review, Holt (1997) presents a series of novel ideas that may provide useful directions for further research. However, the lack of important information regarding the spermatozoon's fundamental cryobiological properties, including osmotic and membrane permeability characteristics, has hindered the progress in alternative strategies such as desiccation, vitrification and freeze drying of long-term storage of bovine spermatozoa.

Besides being exposed to subphysiological pH and supraphysiological osmolarity, oxygen levels in the epididymis are much lower than in air. It is generally assumed that oxygen could play a role in reducing sperm quality during liquid storage (Maxwell and Stojanow, 1996). During respiration, incomplete reduction of dioxygen results in the formation of reactive oxygen species (ROS) such as hydrogen peroxide, superoxide anion and hydroxyl radical. Increased levels of these extracellular ROS can result in lipid peroxidation of the sperm membranes. However, the importance of reduced oxygen levels for sperm storage has recently been questioned since it was shown that spermatozoa may

lose their fertility regardless of the presence or absence of oxygen (Krzyzosiak et al., 2001). This lack of effect of storage atmosphere in sperm preservation was also obvious from our study in which no significant differences were observed in motility, membrane integrity, mitochondrial membrane potential or DNA integrity of spermatozoa stored either under nitrogen-gassed or aerobic storage conditions **(chapter 4)**. We also found that addition of catalase, a scavenger of ROS, does not improve the fertilizing ability of bull spermatozoa stored at 4°C (Verberckmoes et al., in preparation). Many of the classical antioxidants that are perfectly effective against extracellular oxidative attack (such as catalase) are ineffective for the protection of spermatozoa against intracellular ROS which are generated by mitochondria and could lead to progressive decrease in mitochondrial function, sperm motility and DNA integrity (Twigg et al., 1998). In this respect, the search for scavengers that are able to permeate biological membranes easily and that counteract the intracellular ROS or the search for the mechanism which can reduce the level of endogenous phosphorylation, could have practical implications for improving storage of spermatozoa.

Packing spermatozoa at high concentrations as it occurs in the epididymis was detrimental for sperm viability. The lack of certain nutrients (such as glucose or fructose) in Hepes-TALP and the inability to remove toxic waste products, which are harmful to remaining viable spermatozoa, could probably explain why at lower sperm concentrations the percentage of membrane-intact spermatozoa with a high mitochondrial membrane potential was higher than when spermatozoa were stored at higher concentrations. In the epididymis, nutrients are limited to easily metabolizable substrates and toxic products are disposed of systematically. However, for economical and practical reasons, it is not possible to establish a dynamic in vitro sperm storage system. An alternative method could be to exchange the medium at the moment when motility parameters decrease significantly. A positive effect of medium exchange on recovery of fertile dog semen has already been observed (Iquer-ouada and Verstegen; 2001). It would be very interesting to know whether this is also beneficial for bovine sperm preservation. In conclusion, mere mimicking of epididymal physicochemical conditions in a simple medium such as Hepes-TALP was obviously not efficient as an approach to reversibly immobilize bovine spermatozoa and maintain their fertilizing capacity for several days.

Therefore, the effect of the unique ionic composition of cauda epididymal plasma on the regulation of sperm motility and viability was also investigated. There have been numerous studies examining the effect of different epididymal constituents (Crabo, 1965;

Cascieri et al., 1976; Carr and Acott, 1984; Setchell et al., 1993). However, mimicking the ion concentration which prevails in epididymal plasma (high [K^+], low [Na^+], low [Ca^{2+}] and high [Mg^{2+}]) has not reversibly immobilized mammalian spermatozoa. This may be explained by the fact that most studies have tested only one ion at a time, rather than investigating the overall interaction of principal ions on sperm quality and fertilizing capacity of stored spermatozoa. In our laboratory, research is going on, in which a new completely defined diluent has been developed, based on the composition of cauda epididymal plasma (CEP-diluent: Verberckmoes et al., in preparation). Before the CEP-diluent can be applied in the field, its efficiency in preserving sperm viability needs to be tested in vitro. From preliminary results, it could be concluded that after 6 days of storage in CEP-diluent at 4°C spermatozoa were still able to penetrate oocytes (penetration rate = 67%) **(chapter 8)**. In comparison with other diluents for storage of bull spermatozoa (CAPROGEN® and Triladyl®) the linear motility was better preserved when spermatozoa were stored in CEP-diluent (Verberckmoes et al., in preparation). This is important because linear motility is a sperm characteristic, which is correlated to the in vivo fertility of spermatozoa (Zhang et al., 1998). Despite the fact that the CEP-diluent was successful for maintaining sperm penetrating ability for up to 6 days, our experiments were still unsuccessful in demonstrating the mechanism responsible for reversible suppression of sperm motility and prolonged viability as observed in vivo. This could mean that mimicking the unique ionic composition of cauda epididymal plasma is not sufficient to reversibly suppress sperm motility.

Another approach could be the use of a geometric method to search the best medium for sperm preservation. Such a geometric method has already been used to study the joint effects of different components on the development of outbred CF1 mouse zygotes to the blastocyst stage (Lawitts and Biggers, 1992) resulting in a successful Simplex Optimized Medium for the culture of embryos.

Another possibility for the preservation of spermatozoa may be found in the addition of epididymis-secreted proteins, which play an important role in sperm protection in vivo. Sperm binding proteins secreted uniquely by cauda epididymal epithelial cells, could be interesting candidates for the survival of spermatozoa, while caput-specific proteins may be important for sperm maturational changes (Hinton and Palladino, 1995). However, despite the fact that we were able to culture caput and cauda epididymal epithelial cells in vitro which secreted hormone- and region-dependent proteins

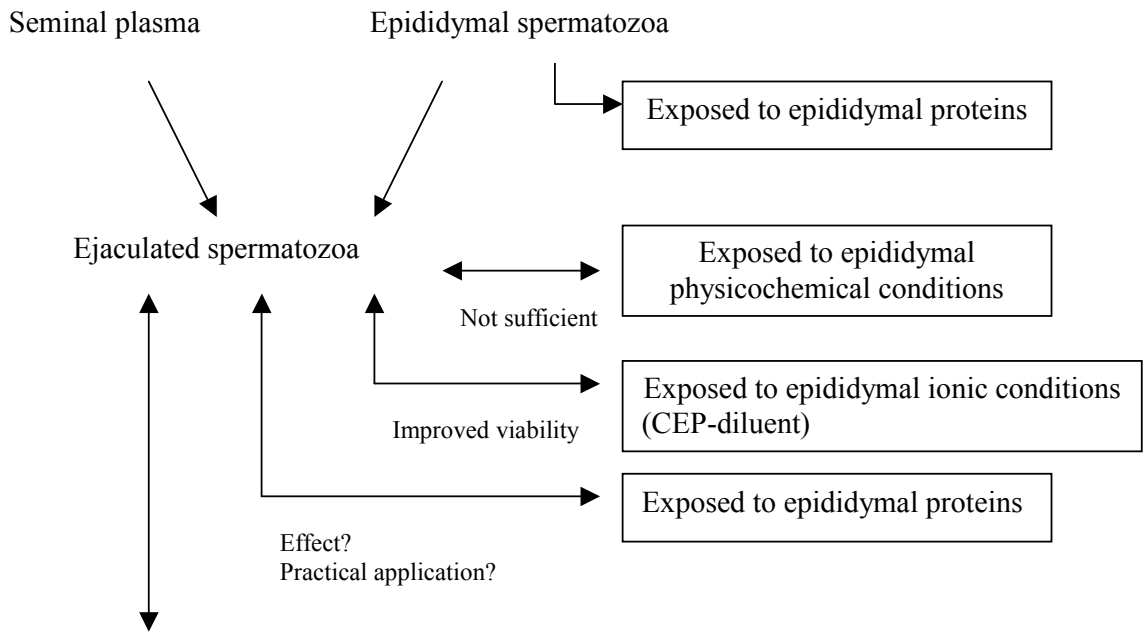
(chapter 6), it was not possible to determine specific proteins regulating sperm viability. We decided not to proceed with this research for the following two reasons. Firstly, all our experiments were performed with ejaculated bull spermatozoa instead of epididymal spermatozoa because ejaculated spermatozoa can be obtained in large numbers while AI with epididymal bull spermatozoa only happens on very rare occasions (Foote, 2000). Ejaculated spermatozoa differ from epididymal spermatozoa due to their interaction with secretions of the accessory sex glands at the time of ejaculation (Baas et al., 1983; Shannon et al., 1987). Several bovine seminal plasma proteins (collectively called BSP-proteins) are known to bind to the sperm surface inducing plasma membrane changes by stimulating cholesterol and phospholipid efflux **(*figure 1A*)**. Since cholesterol is known to have a stabilizing effect on membranes (Yeagle, 1985), this efflux causes destabilization of the membrane and results to premature capacitation. Therefore, it would be difficult to "turn back the clock" for ejaculated spermatozoa. This means that some specific membrane-stabilizing epididymis-secreted proteins become of minor importance for the viability of spermatozoa once they are ejaculated. This hypothesis can be elucidated by an example in the human where the epididymal protein HE1 is involved in maintaining the high cholesterol content of spermatozoa during epididymal transit and storage. However this cholesterol carrier protein does not bind firmly to ejaculated spermatozoa (Kirchhoff, 1998) making it impractical to use for increasing membrane stability.

Figure 1: Proposed model for events that may occur in (A) non-diluted spermatozoa (B) conventionally diluted spermatozoa and (C) 'coated' diluted spermatozoa, when they bind to oviduct explants. (A) Non-diluted spermatozoa are exposed to BSP proteins during both ejaculation and storage. These BSP proteins bind to choline phospholipids of the sperm membrane resulting in the efflux of choline phospholipids and cholesterol causing destabilization of the sperm plasma membrane. This leads to a fast decrease in the percentage of viable spermatozoa able to attach to oviduct explants via BSP proteins bound on the sperm membrane. (B) Conventionally diluted spermatozoa are exposed to BSP proteins during ejaculation but not during storage in egg yolk containing diluent. These BSP proteins bind to the sperm plasma membrane, resulting in the efflux of choline phospholipids and cholesterol from the sperm plasma membrane causing destabilization, or bind to low-density proteins (LDL) of egg yolk. During storage in egg yolk containing diluent, LDL of egg yolk compete with BSP proteins in their binding to the sperm membrane and protect the membrane against destabilization. This leads to a high percentage of viable spermatozoa, which are still able to attach to oviduct explants via BSP proteins bound directly or indirectly via LDL on the sperm membrane (C) 'Coated' diluted spermatozoa are not exposed to BSP proteins during ejaculation and storage in egg yolk containing diluent. LDL sequester most of the BSP proteins present in semen and prevent their binding to the sperm membrane. The sperm membrane is 'coated' with LDL of egg yolk resulting in a very high percentage of viable spermatozoa, which are still able to attach to oviduct explants via BSP proteins bound indirectly via LDL on the sperm membrane.

A second reason for not proceeding with this research is the fact that isolation and purification of epididymal proteins, and their immunolocalization on the sperm membrane are labour-intensive and requires a high equipment cost.

Studies examining the effect of seminal plasma on sperm function are contradictory, suggesting both beneficial (motility-stimulating factors: Acott and Hoskins, 1978; Baas et al., 1983, and decapacitationary factors or acrosome-reaction inhibitory factors: Chang, 1957; Dostalova et al., 1994) and detrimental effects (al-Somai et al., 1994; Vishwanath and Shannon, 1997). However, most studies agree that prolonged exposure to seminal plasma markedly reduces the viability and fertilizing capacity of spermatozoa (Vishwanath and Shannon, 1997). Furthermore, several studies reported the minor importance of seminal plasma for the fertilizing ability of spermatozoa. This is demonstrated by the fact that epididymal spermatozoa can successfully be used for insemination in a number of species including the cow (Amann and Griel, 1974). As mentioned above, accelerated cell damage is mainly the consequence of exposure to a group of acidic heparin-binding proteins (BSP proteins), which represents the major protein fraction of bovine seminal plasma. These proteins bind to choline phospholipids of the sperm membrane at ejaculation (Desnoyers and Manjunath, 1992) resulting in the efflux of choline phospholipids (Therien et al., 1999) and cholesterol (Therien et al., 1998) from the sperm plasma membrane causing destabilization of the membrane **(*figure 1A*)**. Dott et al. (1979) have suggested that this toxic effect is immediate and persists even after washing the spermatozoa. More recent studies by Muller et al. (1998) have demonstrated the incorporation of BSP proteins into the sperm membranes.

Exposure of spermatozoa to liposomes with cholesterol could be an option to increase membrane stability, and delay capacitation and induction of acrosome reaction (Parks and Ehrenwald, 1990). However the procedure for making cholesterol liposomes is very expensive and time-consuming which makes it less useful for practical applications. Another and more economical option to maintain sperm viability is the quick removal of seminal plasma (Way et al., 2000). Centrifugal sedimentation of semen and resuspension of spermatozoa in fresh medium is generally accepted to be the quickest and most effective method of removing seminal plasma, however this method causes cell damage (Harrison and White, 1972) **(*figure 2*)**.



		Advantage	Disadvantage
1.	Centrifugation	fast and easy	cell damage
2.	Dilution of semen in egg yolk containing diluent "sperm coating"	fast, easy and no cell damage	risk for contamination when stored at RT
3.	Addition of liposomes containing cholesterol/phospholipids	no risk for contamination	complicated and expensive

Figure 2: Schematic representation of the different approaches used in this study to prolong sperm viability in vitro

This encouraged us to search for a new efficient method to remove seminal plasma as quickly as possible without damaging spermatozoa. Simultaneously with the observation of Manjunath et al. (2002), we have found that the detrimental effects of BSP proteins could be minimized by dilution with egg yolk containing diluent within minutes of semen collection (*Figure 1B*). Our aim was to limit the contact time between spermatozoa and seminal plasma even more (within seconds) by collecting bull semen directly in an egg yolk containing diluent during ejaculation (***figure 1C***). In this respect, the BSP protein-binding capacity of low-density lipoproteins (LDL) of egg yolk may represent an important mechanism of sperm protection. This binding happens quickly, is specific, saturable and stable even after freeze-thawing or ultracentrifugation of semen (Manjunath et al., 2002). Low-density lipoproteins sequester most of the BSP proteins present in semen and prevent

their binding to the sperm membrane **(*figure 1C*)**. Vishwanath et al. (1992) suggest that LDL compete with detrimental seminal plasma cationic peptides (< 5kDa) in binding to the sperm membrane.

In our study, the beneficial effect of sperm "coating" during ejaculation is expressed in a significantly higher recovery of membrane-intact spermatozoa with a high mitochondrial membrane potential, and a significantly higher oocyte penetration rate after 4 days of storage in Hepes-TALP (pH 7) at RT in comparison to "control" spermatozoa **(chapter 5, *figure 3*)**. However, the motility apparatus was not protected by sperm "coating" when stored in Hepes-TALP (pH 7) at RT.

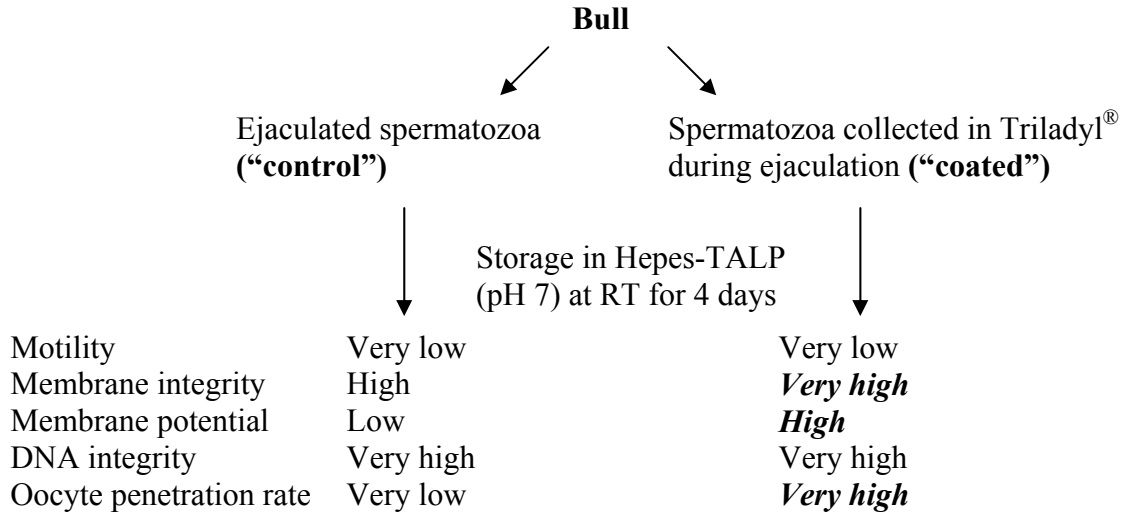


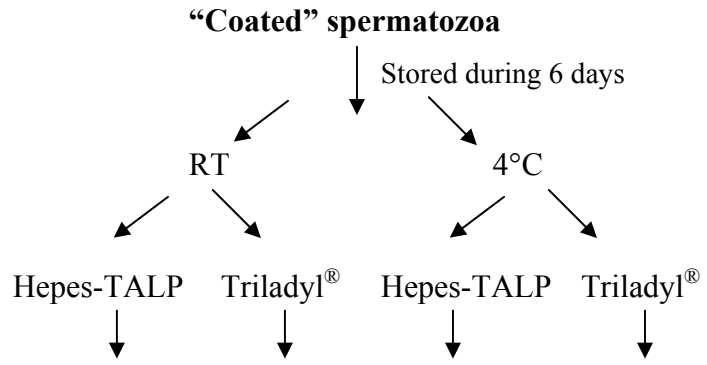
Figure 3: Schematic representation of sperm characteristics of "control" and "coated" spermatozoa stored at a concentration of 10 x 10^6 spermatozoa/ml in Hepes-TALP (pH 7) for up to 4 days. (Very low: < 20%; low: 20-50%; high: 50-70%; very high: > 70%)

Interestingly, no decrease in sperm quality was observed when "coated" spermatozoa were stored in an egg yolk containing diluent (Triladyl®) at RT for up to 6 days. In this case, the protective action of egg yolk prevented a decrease in progressive motility, membrane integrity and in mitochondrial membrane potential of spermatozoa, resulting in a significantly higher oocyte penetration rate after 6 days of storage in Triladyl® at RT than in Hepes-TALP at pH 7 **(*figure 4*)**. As mentioned above, it is obvious that the composition of the storage medium and even the method of sperm collection may have pronounced effects on sperm survival in vitro.

Another factor that influences the viability of spermatozoa after ejaculation is the storage temperature. At normal body temperature, sperm viability in vitro is limited to a few hours for most species. It is caused by the fact that spermatozoa have very limited

biosynthetic activity (Hammerstedt and Andrews, 1997). Spermatozoa must be deep-frozen below -130°C to halt their metabolic processes, so that thermally driven chemical reactions cannot take place (Medeiros et al., 2002). However, the freezing and thawing processes cause irreversible damage to a large proportion of bull spermatozoa and render the remainder more sensitive to environmental stress (Hammerstedt et al., 1990; Parks and Graham, 1992; Shannon and Vishwanath, 1995). This kind of long-term preservation was no option in our study and therefore, semen stored at RT or at 4°C was used as an alternative for frozen-thawed semen.

From our results it was clear that semen stored at 4°C in Hepes-TALP (pH 7) during 4 days showed a very low oocyte penetration capacity in comparison to semen stored in Hepes-TALP (pH 7) at RT (***figure 4***). This difference in oocyte penetration rate could not be explained by the decline in motility of stored spermatozoa since it was low in both groups. It is probably due to destabilization of the sperm membranes, as a consequence of cold shock injuries, inducing capacitation or acrosome reaction.



Total motility	Low	Very high	Low	Very high
Progressive motility	Very low	Very high	Very low	Very high
Membrane integrity	High	Very high	Very high	Very high
Membrane potential	High	Very high	Very high	Very high
Oocyte penetration rate	High	Very high	Very low	Very high

Figure 4: Schematic representation of sperm characteristics of "coated" spermatozoa stored at a concentration of 10 x 10^6 spermatozoa/ml in Hepes-TALP (pH 7) or in Triladyl® at RT or at 4°C for up to 6 days. (Very low: < 20%; low: 20-50%; high: 50-70%; very high: > 70%)

Cold shock injury of spermatozoa can occur when ejaculated semen is very quickly cooled down to temperatures below 16°C (Halangk et al., 1982). Therefore, it is evident that the protection of sperm membranes in order to render them more stable at reduced temperatures would improve sperm viability. Egg yolk, a common constituent of most semen diluents, is recognized to increase sperm fertilizing ability in vivo when present in diluents for sperm storage at RT (Shannon and Curson, 1983; Barak et al., 1992). Moreover, egg yolk appears to prevent sperm damage during cooling and freezing (Moussa et al., 2002). In our study, the protective action of egg yolk was obvious from the observation that a significantly higher oocyte penetration rate was seen when spermatozoa were stored for up to 6 days in Triladyl®-diluent at both 4°C and RT than in Hepes-TALP. Furthermore, no significant decrease was observed in progressive motility, membrane integrity or mitochondrial membrane potential of spermatozoa when stored in Triladyl® at both temperatures **(*figure 4*)**.

However, whereas cooling to 4°C induces cold shock injury to spermatozoa, which can be intercepted by the addition of egg yolk to the sperm diluent, sperm storage at RT leads to another kind of problem. Bacterial contamination is common when spermatozoa are stored in egg yolk containing media at RT, even in the presence of antibiotics. Therefore, in the future, all experiments will be performed at 4°C because at this temperature the multiplication of pathogens is reduced and addition of egg yolk protects the sperm membranes sufficiently against cold shock. The precise mechanism through which egg yolk protects spermatozoa against cold shock is still unknown but it is largely presumed to be due to LDL (Moussa et al., 2002). Foulkes (1977) suggested that these LDL could adhere to the cell membranes resulting in sperm membrane preservation.

From ***figure 2*** it is clear that improved sperm viability can be obtained after storage in CEP-diluent, and by collecting spermatozoa in an egg yolk containing diluent. These approaches could be used as a potential alternative for preserving sperm quality and fertility. Further research can be done both at the examination of the protein profile of the plasma membrane of "coated" ejaculated spermatozoa and at the comparison of these results with the plasma membrane protein profile of epididymal and "uncoated" ejaculated spermatozoa. A better understanding of the mechanism by which LDL of egg yolk contributes to the protection of spermatozoa during the freeze-thaw process could lead to its replacement by a well-defined substitute of non-animal origin (Moussa et al., 2002). Also for the AI-industry, it would be interesting to know if 'coated' spermatozoa are less

sensitive to the freezing process so that a higher recovery of viable spermatozoa could be obtained after thawing of the sperm sample.

The efficiency of collecting spermatozoa in an egg yolk containing diluent and the quality of CEP-diluent need to be tested in vitro by means of sperm function tests before they can be applied in the field. Since fertilization is a process that requires several sperm functions, it seems logical that several sperm characteristics such as membrane integrity, capacitation status, acrosomal integrity, mitochondrial function, DNA integrity and also more specific characteristics of sperm motility must be considered in order to adequately define "sperm quality". Spermatozoa must be of superior quality in order to migrate to the site of fertilization successfully, bind to and penetrate the zona pellucida and fuse with the oolemma.

For an accurate estimation of "sperm quality", several methods have been developed over the years to test different sperm functions, some of which have been correlated to field fertility **(chapter 3)**. To obtain a valid test for predicting in vivo fertility the following criteria need to be fulfilled 1) a fast objective technique; 2) a sufficient number of sperm samples from different males, and 3) reliable field fertility data. Development of tests, capable of analyzing more than one sperm function simultaneously, remains an important objective (Muller, 2000).

In vitro fertilization of zona-intact homologous eggs is at present the most informative test of sperm fertilizing ability. However, there are still pronounced differences between in vitro and in vivo fertilization. For in vivo fertilization, movement of spermatozoa in the genital tract is necessary and conditions of sperm capacitation and sperm:egg ratios at the site of fertilization are completely different from the situation in vitro. Furthermore, only one spermatozoon can be evaluated per oocyte, which makes oocyte penetration an all or non event. The number of oocytes tested per bull should therefore be in the order of several hundred so that any differences between animals can be measured. Another problem is the variability of oocyte maturation between different oocyte batches, which contributes substantially to the variation between tests (den Daas, 1997). Moreover, some spermatozoa retain the ability to penetrate the oocyte even after they are damaged to such extent that they are able to fertilize but unable to induce correct cleavage and development of fertilized oocytes (Krzyzosiak et al., 2001). This means that oocyte cleavage would be a better indicator of sperm quality than oocyte penetration rate and than blastocyst development, which is largely dependent on culture conditions (Zhang et al., 1997). Taking into account these disadvantages and the fact that IVF is a very

expensive and time-consuming procedure, its routine use as an exclusive indicator of bull fertility cannot be recommended. The search for an alternative test which is simple and economical must continue.

Our hypothesis is that a sperm-oviduct binding assay could be a potential bio-assay to evaluate several of the prerequisites that spermatozoa must have in order to achieve fertilization. Previous studies have already shown that attachment of spermatozoa to oviduct explants in co-culture is a process that selects higher quality spermatozoa from a given sample, including morphologically normal and motile spermatozoa with intact plasma and acrosomal membranes and higher quality chromatin structure (Thomas et al., 1994; Ellington et al., 1999). In addition, bull spermatozoa selectively attached to oviduct epithelial cells supported significantly superior embryo cleavage and embryonic development rates than spermatozoa that did not attach to oviduct epithelial cells (Ellington et al., 2000).

Chapters 7 and **8** describe a fast and reproducible sperm-oviduct binding assay to quantify the number of frozen-thawed spermatozoa by means of fluorescent microscopy and an image database program. Using this standardized assay, a clear difference was observed in sperm binding capacity among individual bulls and evidence was obtained that sperm ability to bind to oviduct explants is related to in vivo fertility of the sperm donor **(chapter 7)**. A statistical model **(figure 4 of chapter 7)** is designed to predict in vivo fertility, as expressed by NRR, of the given sperm sample by means of the number of attached spermatozoa. The accuracy of this model can be improved by testing a higher number of ejaculates originating from bulls with a wide range of in vivo fertility.

However, for practical reasons, the results were based on only one batch of frozen-thawed semen from 10 Holstein Friesian bulls with known non-return rates varying from 52.8% to 69.9%. Significant variation in field fertility between different ejaculates of a single bull can be observed (Zhang et al., 1997). Therefore it is risky to assess the temporal fertility of a bull based on the results of only one frozen semen batch. This ejaculate effect on results can be minimized by testing different ejaculates or by pooling spermatozoa from different ejaculates from the same bull in one test. Most studies investigating the relationship between sperm quality and in vivo bull fertility were performed with less than 20 bulls **(chapter 3)**. Using our approach, it is possible to test more bulls during the course of an experiment. We are not restricted to the use of the same oviduct explant batch, since in **chapter 8** no effect of oviduct explant batch on sperm binding capacity was observed. It is obvious that a significant correlation between in vivo fertility and in vitro test results can

only be obtained if the range of in vivo fertility is large. However, variation in NRR between bulls that are used for AI is generally in the order of 10 to 15% (Uwland, 1984). In our study the widest possible range in NRR of bulls available from the AI center was 17%. A wide range in fertility is difficult to obtain, since bulls that have been preselected as having inadequate semen characteristics after collection are not evaluated for field fertility. The accuracy of field fertility data also had a profound effect on the interpretation of the results. It is well known that factors related to sire, cow, inseminator, season and herd health management influence field fertility (de Kruif, 1978; den Daas, 1997). Therefore, in our study only sires of the same age with comparable numbers of services, and cows inseminated by experienced inseminators during autumn and winter were used. In conclusion, further refinement of our in vitro model is needed before it can be concluded whether the sperm-oviduct binding assay is a reliable tool to predict in vivo bull fertility.

From the results of **chapter 8** it is clear that the sensitivity of the sperm-oviduct binding assay was comparable to that of the oocyte penetration assay for the assessment of sperm quality of cooled bovine spermatozoa stored in CEP, CAPROGEN® or Triladyl® for up to 6 days. It was not possible to investigate the correlation between sperm-oviduct binding assay and in vivo fertility of cooled semen. For practical reasons, it is not feasible to obtain data of NRR after insemination with liquid spermatozoa from different bulls in Belgium. This can only be performed in New Zealand, where a unique system of seasonal breeding exists. In this country more than 85% of all inseminations are performed with liquid stored semen during spring and summer (Vishwanath et al., 1996; Vishwanath and Shannon, 2000). Furthermore, it is also difficult to use the same ejaculate for both AI and sperm-oviduct binding assay.

Concluding remarks

From the results described in this thesis, four conclusions can be drawn:

1. improved sperm quality can be obtained by collecting semen within seconds after ejaculation in egg yolk containing diluent. This approach could be used as potential alternative for preserving sperm quality and in vivo bull fertility.
2. despite the fact that we were able to culture caput and cauda epididymal epithelial cells in vitro which secrete hormone- and region-dependent proteins, it was not possible to determine specific proteins that regulate sperm viability.
3. our newly developed sperm-oviduct binding assay could become a promising functional test for the assessment of frozen-thawed sperm quality.
4. the sensitivity of the sperm-oviduct binding assay for the assessment of sperm quality of cooled bovine spermatozoa was comparable to that of the oocyte penetration assay.

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10.

SUMMARY / SAMENVATTING

Summary

The goal of the cattle AI-industry is to maximize the number of offspring produced by selected genetically superior bulls. This is accomplished by maximizing the number of inseminated cows per ejaculate without a reduction in fertility. The ability of spermatozoa to fertilize when inseminated depends mostly upon its quality after storage. In this respect, both biochemical composition of the diluent and storage temperature are key factors affecting the lifespan of spermatozoa during storage. In the 20th century, two major storage technologies for liquid and frozen semen were developed, both with advantages and disadvantages **(chapter 1)**.

The general aims of this thesis were to maintain the quality and fertilizing ability of stored bovine spermatozoa by means of knowledge obtained by investigating different physicochemical conditions, and ionic and protein composition of cauda epididymal plasma **(chapter 4, 5** and **6)**, and to set up a new sperm-oviduct binding assay in order to use it as a new sperm function test for the prediction of in vivo bull fertility **(chapter 7** and **8)**.

In **chapter 2**, the role of the epididymis for sperm conservation in vivo is described. Special attention is given to the transport, maturation and storage of spermatozoa in the epididymis, and to the biochemical composition of cauda epididymal plasma.

In **chapter 3**, different methods for the evaluation of sperm quality such as fluorescent dyes, motility assessment assays, and sperm binding and performance assessment assays are discussed.

Ejaculated spermatozoa diluted in vitro remain viable only for a limited period of time. Despite the development of numerous diluents for storage at 4°C or at room temperature (RT), it is still not possible to preserve the fertilizing potential of spermatozoa longer than 3 to 5 days. In **chapter 4**, an experiment is described in which ejaculated bull spermatozoa were stored in the simple salt solution Hepes-TALP at RT for up to 4 days. This medium was modified to obtain physicochemical conditions similar to those prevailing in the cauda epididymidis. The effect of change in pH (4-8) or change in osmolarity (100-800 mOsm) of Hepes-TALP, storage atmosphere (nitrogen-gassed or aerobic) and sperm concentration (10 x 10^6-1 x 10^9 spermatozoa/ml or undiluted) on

different sperm characteristics such as membrane integrity, motility, mitochondrial membrane potential or DNA integrity of the sperm sample was investigated in order to assess sperm quality after storage. From the results it is clear that decreasing the pH of the medium by addition of the strong acid HCl, increasing the osmolarity by addition of sorbitol and storage of highly concentrated spermatozoa under nitrogen-gassed conditions are not effective methods for long-term preservation of spermatozoa in vitro. In this respect, it was not possible to mimic the luminal environment of the bovine cauda epididymidis in order to store densely packed bovine spermatozoa in a potentially fertilizing condition for several days after ejaculation. The main problems were the lack of certain nutrients such as glucose or fructose in Hepes-TALP and the inability to remove toxic waste products which are harmful for remaining viable spermatozoa.

In **chapter 5**, an attempt was made to maintain the quality of stored spermatozoa by protecting them against detrimental bovine seminal plasma (BSP) proteins. These proteins are known to bind to choline phospholipids of the sperm membrane at ejaculation resulting in the efflux of choline phospholipids and cholesterol and causing destabilization of the sperm plasma membrane. In our approach, bull semen was collected in egg yolk containing diluent during ejaculation, which made it possible to limit the contact time between spermatozoa and seminal plasma within seconds. The BSP protein-binding capacity of the low-density lipoproteins (LDL) of egg yolk may represent an important mechanism of sperm protection. In this respect, LDL sequester most of the BSP proteins present in semen and prevent their binding to the sperm membrane. In our study, the beneficial effect of LDL of egg yolk is expressed as a significantly higher recovery of membrane-intact spermatozoa with a high mitochondrial membrane potential, and in a significantly higher oocyte penetration rate even after storage in Hepes-TALP at pH 7 at RT for 4 days. When these “coated” spermatozoa were stored for longer than 4 days at RT or at 4°C, it remained necessary to protect spermatozoa by addition of egg yolk to the storage medium. The higher oocyte penetration rates of stored spermatozoa obtained by sperm “coating”, makes our approach a potential alternative for preserving sperm quality and fertility.

Specific hormone-dependent protein secretions from epididymal principal cells are associated with spermatozoa during their maturation and storage and play a fundamental role in modifying the surface characteristics of spermatozoa in preparation for the events of

fertilization. Therefore, in **chapter 6**, the effect of hormones (testosterone, dihydrotestosterone and hydrocortisone) on protein secretions of caput and cauda epididymal epithelial cells cultured in principal cell medium (PCM) was determined in order to identify proteins important for sperm survival. A confluent monolayer of caput and cauda epididymal epithelial cells was obtained in serum-containing PCM after 7 days of culture at 38.5°C (5% CO2 in air) in the presence or absence of hormones. Neither epithelial detachment nor substantial overgrowth of fibroblasts were observed during 10 days of culture. The secreted proteins were separated by means of 2D-SDS-PAGE. Comparison of the different protein patterns showed 61 spots in total, 11 of which were only secreted in the presence of hormones, 3 of which appeared to show hormone-related changes and 25 of which were caput or cauda specific. Most of these secreted proteins were low molecular weight acidic proteins. More than 60% of these proteins present in caput or cauda cultures correspond to proteins secreted in caput or cauda epidiymal plasma, which confirms their epididymal origin. Despite the fact that we were able to culture caput and cauda epididymal epithelial cells in vitro which secrete hormone- and region-dependent proteins it was not possible to determine specific proteins that regulate sperm viability. Additional research is needed to identify and isolate specific proteins and investigate their immunolocalization on the sperm membrane. Furthermore, it is possible that some specific membrane-stabilizing epididymis-secreted proteins are of minor importance to the viability of ejaculated spermatozoa, which differ from epididymal spermatozoa due to their interaction with secretions of the accessory sex glands at the time of ejaculation.

Fertilization is a complex process that requires several sperm functions. Therefore it seems logical that different sperm characteristics such as membrane integrity, capacitation status, acrosomal integrity, mitochondrial function, DNA integrity and also more specific characteristics of sperm motility must be considered in order to adequately define “sperm quality”. To be able to estimate “sperm quality” accurately, several sperm function assays have been developed over the years to test different aspects of semen quality, some of which have been correlated to field fertility **(chapter 3)**. However, more attention is now given to the development of tests analyzing more than one sperm function at the same time. Our hypothesis is that a sperm-oviduct binding assay could be a potential bio-assay to evaluate several of the prerequisites that sperm must have in order to achieve fertilization. In **chapter 7**, an in vitro bio-assay was set up to quantify the number of

frozen-thawed spermatozoa bound to oviduct explants in order to determine whether this assay could be used to predict in vivo bull fertility. Sperm binding capacity was evaluated by counting JC-1-labelled spermatozoa attached to defined oviduct explants. From the results it is clear that Hepes-TALP is a more useful medium than IVF-TALP, TCM-199 + 10% FCS and TCM-199 for the investigation of sperm binding to oviduct explants, and that oviduct explants with a surface smaller than 20 000 μm² provided more consistent results than explants with a surface larger than 100 000 μm². Using this bio-assay, differences were observed in the sperm binding capacity among individual bulls. Evidence was obtained that sperm binding capacity to oviduct explants is related to in vivo fertility of the sperm donor, when measured after 24 h of sperm-oviduct culture, and provided that the membrane integrity of the sperm sample was higher than 60%. Further refinement of our in vitro model, by testing different ejaculates from more bulls, is needed before it can be concluded whether the sperm-oviduct binding assay is a reliable tool to predict in vivo bull fertility.

The sperm-oviduct binding assay was further refined in order to make it possible to test more bulls during the course of an experiment. We found that we are not restricted to the use of the same oviduct explant batch, since in **chapter 8** no difference on sperm binding capacity was observed between different batches of high quality oviduct explants. This sperm-oviduct binding assay was then used to analyze the oviduct binding capacity of cooled bovine spermatozoa stored in three different diluents (CEP, CAPROGEN® and Triladyl®) for up to 2, 4 and 6 days (D_2-, D_4- and D_6-spermatozoa). No significant effect of diluent on sperm-oviduct binding capacity of D_2-, D_4- and D_6-spermatozoa was observed after 30 min, 24 and 48 h of co-culture. In an additional experiment, it was shown that oocyte penetration rate of cooled bovine spermatozoa stored for up to 6 days was also not significantly affected by diluent type. This means that the sensitivity of the sperm-oviduct binding assay was comparable to that of the oocyte penetration assay for the assessment of sperm quality of cooled bovine spermatozoa stored in CEP, CAPROGEN® or Triladyl® for up to 6 days.

The general discussion and conclusions are presented in **chapter 9**.

From the results described in this thesis, four conclusions can be drawn:

1. improved sperm quality can be obtained by collecting semen within seconds after ejaculation in egg yolk containing diluent. This approach could be used as potential alternative for preserving sperm quality and in vivo bull fertility.
2. despite the fact that we were able to culture caput and cauda epididymal epithelial cells in vitro which secrete hormone- and region-dependent proteins, it was not possible to determine specific proteins that regulate sperm viability.
3. our newly developed sperm-oviduct binding assay could become a promising functional test for the assessment of frozen-thawed sperm quality.
4. the sensitivity of the sperm-oviduct binding assay for the assessment of sperm quality of cooled bovine spermatozoa was comparable to that of the oocyte penetration assay.

Samenvatting

Eén van de doelstellingen van kunstmatige inseminatie (KI) is het verkrijgen van een maximaal aantal nakomelingen per ejaculaat van genetisch hoogwaardige fokstieren zonder dat er een daling van de in vivo fertiliteit optreedt. De slaagkansen op bevruchting na KI zijn hoofdzakelijk afhankelijk van de spermakwaliteit. Daarbij zijn zowel de biochemische samenstelling van de verdunner als de bewaartemperatuur de belangrijkste factoren voor de overleving van het sperma. In de loop van de vorige eeuw werden reeds verschillende verdunners voor het invriezen van sperma bij -196°C of voor het bewaren van sperma bij kamertemperatuur (KT) of bij 4°C ontwikkeld. De voor- en de nadelen van het invriezen en bewaren van sperma bij KT of bij 4°C staan beschreven in **hoofdstuk 1**.

De studie had twee doelstellingen:

- het verlengen van de levensduur van stierensperma door het effect van fysisch-chemische condities, en de ionen- en proteïnensamenstelling van het epididymaal plasma op de overleving van spermatozoa na te gaan (**hoofdstuk 4, 5 and 6**).
- het op punt stellen van een nieuwe in vitro spermafunctietest om de in vivo fertiliteit van stieren te kunnen voorspellen (**hoofdstuk 7 and 8**).

In **hoofdstuk 2** wordt een overzicht gegeven van het transport, de rijping en de bewaring van spermatozoa in de epididymis, en van de biochemische samenstelling van het epididymaal plasma.

In **hoofdstuk 3** worden de verschillende methodes besproken die gebruikt worden voor de evaluatie van de spermakwaliteit zoals fluorescerende kleurstoffen, motiliteitsbepalingen, spermabindingstesten en in vitro fertilisatie experimenten.

Geëjaculeerde spermatozoa hebben slechts een zeer korte levensduur. Ondanks de ontwikkeling van verschillende verdunners voor de bewaring van spermatozoa bij 4°C of bij KT, is het tot op heden niet mogelijk gebleken om het bevruchtend vermogen van geëjaculeerde spermatozoa van de stier te verlengen tot meer dan 3 dagen. In **hoofdstuk 4** wordt een onderzoek beschreven voor het bewaren van geëjaculeerde spermatozoa gedurende 4 dagen in een eenvoudige zoutoplossing Hepes-TALP bij KT. Het effect van de pH (4-8) en van de osmolariteit (100-800 mOsm) van het medium, van de atmosfeer (stikstof of aëroob) en van de spermaconcentratie (10×10^6-1×10^9 spermatozoa/ml of

onverdund sperma) op de spermakwaliteit werd onderzocht. Hiertoe werden verschillende spermakarakteristieken zoals membraanintegriteit, beweeglijkheid, mitochondriale membraanpotentiaal en DNA-integriteit van het spermastaal geëvalueerd. Uit de resultaten kan besloten worden dat een daling van de pH van het medium door toevoeging van HCl, een stijging van de osmolariteit van het medium door toevoeging van sorbitol, een doorgassing van het medium met stikstof of de bewaring van sterk geconcentreerde spermatozoa geen doeltreffende methoden zijn om de overleving van spermatozoa te bevorderen. Uit dit onderzoek bleek dat het op deze manier niet mogelijk was om de leefbaarheid van hooggeconcentreerde spermatozoa gedurende verschillende dagen te verlengen. De grootste problemen waren de afwezigheid van de nodige voedingsstoffen in Hepes-TALP en het onvermogen om toxische afvalproducten te verwijderen.

In **hoofdstuk 5** wordt beschreven hoe op een alternatieve en meer praktische manier de overleving van stierensperma verbeterd kan worden. Dit gebeurde door spermatozoa zowel tijdens als na de ejaculatie te beschermen tegen de schadelijke 'bovine seminal plasma' (BSP) proteïnen. Deze BSP proteïnen binden aan cholinefosfolipiden van de spermamembraan, waardoor cholinefosfolipiden en cholesterol verwijderd worden en de membraan minder stabiel wordt. Onze methode houdt in dat geëjaculeerde spermatozoa rechtstreeks opgevangen worden in een eidooierbevattende verdunner. Aldus kan het contact tussen spermatozoa en zaadplasma beperkt worden tot enkele seconden. Een belangrijke beschermende eigenschap van eidooier is gebaseerd op de aanwezigheid van lipoproteïnen met een lage densiteit (LDL). Door hun capterend vermogen verhinderen deze LDL de binding van BSP proteïnen aan de spermamembraan. Toepassing van deze nieuwe spermacollectiemethode resulteerde in een significant hoger percentage membraan-intacte spermatozoa met een hoge mitochondriale membraanpotentiaal en in een significant hoger percentage eicellen die gepenetreerd werden door spermatozoa die 4 dagen bewaard werden in Hepes-TALP (pH 7) bij KT. Wanneer deze "gecoate" spermatozoa langer dan 4 dagen bewaard moeten worden bij KT of bij 4°C, is het noodzakelijk om eveneens eidooier toe te voegen aan het medium. De betere spermakwaliteit en het hoger percentage eicelpenetratie verkregen na bewaring van "gecoate" spermatozoa, maakt deze methode zeer waardevol.

Specifieke hormoonafhankelijke proteïnen die gesecreteerd worden door epididymale epitheelcellen binden aan de spermatozoa tijdens hun rijpings- en

bewaarproces in de epididymis en spelen een fundamentele rol in de modificatie van de spermacelmembraan ter voorbereiding van de bevruchting. In **hoofdstuk 6** wordt een onderzoek beschreven naar de invloed van hormonen (testosteron, dihydrotestosteron en hydrocortisone) op de secretie van proteïnen door caput en cauda epididymale epitheelcelculturen. Het was de bedoeling specifieke proteïnen die belangrijk zijn voor de overleving van het sperma te identificeren. Een monolaag van caput of cauda epididymale epitheelcellen in serumbevattend 'Principal Cell Medium' werd verkregen na 7 dagen incubatie bij 38.5°C en 5% CO2 in lucht en dit zowel in de af- als in de aanwezigheid van hormonen. Loskomende epitheelcellen of overgroei met fibroblasten werd niet waargenomen gedurende de eerste 10 dagen na het opstarten van de monolaagcultuur. De gesecreteerde proteïnen werden vervolgens gescheiden door 2D-SDS-PAGE. Na vergelijking van de verschillende proteïnengels werden 61 spots nader bekeken waarvan 11 spots enkel gescreteerd werden in de aanwezigheid van hormonen. Drie spots vertonen hormoonafhankelijke veranderingen en 25 spots werden enkel gescreteerd in de caput of cauda cultuur. De meeste van deze proteïnen hebben een laag moleculair gewicht en een pI-waarde tussen 4 en 6,5. Meer dan zestig procent van de proteïnen die gesecreteerd werden in een caput of cauda cultuur correspondeerde met proteïnen aanwezig in caput of cauda epididymaal plasma waarmee hun epididymale oorsprong werd bevestigd. Ondanks het feit dat we in staat waren een een monolaag van epididymale epitheelcellen te kweken die hormoon- en plaatsafhankelijke proteïnen secreteerde, was het onmogelijk om specifieke proteïnen te vinden die belangrijk zijn voor de overleving van het sperma. Daarvoor is een bijkomend onderzoek nodig teneinde proteïnen te kunnen identificeren, te isoleren en hun localisatie op de spermamembraan te bepalen. Anderzijds moet rekening gehouden worden met het feit dat bepaalde membraanstabiliserende proteïnen die gesecreteerd worden door de epididymis waarschijnlijk minder belangrijk zijn voor de overleving van geëjaculeerde spermatozoa, omdat deze verschillen van epididymale spermatozoa door hun interactie met componenten uit het zaadplasma tijdens de ejaculatie.

Een bevruchting is een gecompliceerd proces waarvoor verschillende spermafuncties vereist zijn. Om het bevruchtend vermogen van een spermastaal zo nauwkeurig mogelijk te kunnen voorspellen is het noodzakelijk om zoveel mogelijk spermakarakteristieken van het te onderzoeken spermastaal na te gaan. Meer bepaald gaat het hierbij om de membraanintegriteit, de capacitatiestatus, de acrosoomstatus, de DNA integriteit en meer specifieke bewegingskarakteristieken van het spermastaal. Al deze

spermakenmerken kunnen bepaald worden aan de hand van specifieke spermafunctietesten: sommigen van deze kenmerken zijn gecorreleerd met de in vivo fertiliteit (**hoofdstuk 3**). Tegenwoordig wordt meer en meer gezocht naar nieuwe spermafunctietesten waarmee verschillende spermakarakteristieken tegelijkertijd geanalyseerd kunnen worden. In **hoofdstuk 7** wordt een nieuwe test beschreven die het mogelijk maakt het aantal ontdooide spermatozoa, van een bepaald ejaculaat, gebonden aan oviductexplanten op een nauwkeurige manier te tellen teneinde de in vivo fertiliteit van de betreffende stier te kunnen voorspellen. Deze spermabindingscapaciteit werd geëvalueerd door het aantal JC-1-gelabelde spermatozoa gebonden aan oviductexplanten met bekende oppervlakte te tellen. Uit de resultaten kan besloten worden dat Hepes-TALP een geschikter medium is voor de sperma-oviduct co-cultuur dan IVF-TALP, TCM-199 en TCM-199 + 10% serum. Tevens verbeterde de herhaalbaarheid van de test wanneer oviductexplanten met een oppervlakte kleiner dan 20 000 µm² werden gebruikt. Aan de hand van deze test kunnen verschillen in spermabindingscapaciteit aan oviductexplanten vastgesteld worden. Bovendien kon bewezen worden dat de spermabindingscapaciteit aan oviductexplanten gerelateerd is met de in vivo fertiliteit van de spermadonor, wanneer bepaald 24 uren na de start van sperma-oviduct co-incubatie, mits de membraanintegriteit van het spermastaal hoger is dan 60%. Een bijkomende verfijning van dit in vitro model is echter nodig (door verschillende ejaculaten van verschillende stieren te testen) om te kunnen besluiten of deze sperma-oviduct bindingstest een betrouwbare methode is om de in vivo fertiliteit van stieren te kunnen voorspellen.

In **hoofdstuk 8** werden explanten afkomstig van verschillende oviducten gebruikt om na te gaan of verschillende oviductexplantculturen kunnen gebruikt worden om sperma van verschillende stieren te testen binnen hetzelfde experiment. Er werd geen significant verschil in spermabindingscapaciteit tussen de oviductexplantculturen gevonden. Aldus is het mogelijk om de resultaten van verschillende experimenten te vergelijken. De sperma-oviductbindingstest werd vervolgens gebruikt om de bindingscapaciteit van stierensperma na bewaring bij 4°C in 3 verschillende verdunners (CEP, CAPROGEN® en Triladyl®) gedurende 2, 4 en 6 dagen (D_2, D_4 en D_6-spermatozoa) te testen. Uit de resultaten werd geen significant effect van de verdunner op de sperma-oviductbindingscapaciteit van D_2, D_4 en D_6-spermatozoa na 30 min, 24 en 48 co-cultuur gevonden. In een bijkomend experiment werd aangetoond dat de eicelpenetratie van sperma bewaard bij 4°C gedurende 6 dagen, eveneens niet significant beinvloed wordt door de gebruikte verdunner. De

conclusie die hieruit getrokken kan worden is dat de sperma-oviductbindingstest even gevoelig is als de eicelpenetratietest voor de evaluatie van de kwaliteit van het sperma dat gedurende 6 dagen bewaard werd in CEP, CAPROGEN® of Triladyl® bij 4°C.

Hoofdstuk 9 bevat de algemene discussie en de conclusies.
De conclusies van dit onderzoek zijn dat:

1. de overleving van het sperma kan verbeterd worden door geëjaculeerde spermatozoa rechtstreeks op te vangen in een eidooierbevattende verdunner. Op deze manier wordt het contact tussen spermatozoa en zaadplasma beperkt tot enkele seconden.
2. niettegenstaande er een monolaag van epididymale epitheelcellen kan worden gekweekt die hormoon- en plaatsafhankelijke proteïnen secreteerde, het onmogelijk was om specifieke proteïnen te vinden die belangrijk zijn voor de overleving van het sperma.
3. de sperma-oviductbindingstest een veelbelovende functietest is voor de evaluatie van de spermakwaliteit.
4. de sperma-oviductbindingstest even gevoelig is als de eicelpenetratietest voor de evaluatie van de kwaliteit van gekoeld stierensperma.

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Ingrid

Curriculum vitae

PERSONALIA

Ingrid De Pauw werd geboren op 24 april 1976 te Gent. In 1994 beëindigde zij haar humaniora-opleiding aan het Visitatiehumaniora te Gent, richting Economische. In 1996 behaalde zij het diploma van kandidaat in de Farmaceutische Wetenschappen en in 1998 het diploma van licentiaat in de Biotechnologie. Onmiddellijk daarna trad zij in dienst als wetenschappelijk medewerker bij de vakgroep Voortplanting, Verloskunde en Bedrijfsdiergeneeskunde op het onderzoeksproject getiteld: "Onderzoek naar diepe inseminatie met lage spermadoses en naar in vivo en in vitro fertiliteit van vers verdund stierensperma." Dit project werd gefinancierd door het Ministerie van Middenstand en Landbouw en de Vlaamse Rundvee Verbetering. In 2003 behaalde zij het getuigschrift van de doctoraatsopleiding in de diergeneeskundige wetenschappen.

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Table 1: Overview of different sperm function tests that have been used in different studies to predict* bull fertility or correlate with it.

Bull Breed	N°	NRR (%) (range)	Number AI/bull (range)	Statistics	Sperm parameter or function (post-thaw)	r	*P*	Reference
SRW[1]	20	50.7 - 74.8	-	Pearson's correlation	Viable and uncapacitated spermatozoa	0.5	$P = 0.03$	Thundathil et al, 1999
HF[2]	13	62.1 - 76.1[†]	250 - 350	Simple and multiple linear regression	Induced acrosome reaction	0.84	$P < 0.005$	Whitfield and Parkinson, 1995*
SRW	15	55 - 83	147 - 882	Linear regression Pearson's correlation	Induced acrosome-reacted spermatozoa with remaining equatorial fluorescence	0.60	$P = 0.0015$	Januskauskas et al., 2000*
					Motility	0.60	$P = 0.0014$	
					Membrane integrity	0.64	$P = 0.0006$	
					Capacitation (B pattern) after swim-up	-0.52	$P = 0.008$	
HF	5		-	Pearson's correlation	Apoptosis: TUNEL (fresh)	-0.9	$P < 0.05$	Anzar et al., 2002
					Viability: Annexin V/PI (fresh)	0.87	$P < 0.05$	
HF	11	70.3 - 74.6	6346 - 59083	Multiple linear regression	Motility parameters (CASA)	Mot: $r^2 = 0.34$	$P < 0.05$	Farrell et al, 1998
						2-5 variables: $r^2 = 0.63$ to 0.98	$P < 0.05$	
HF	5	52.8 - 66.7	198 - 234	Linear regression	Sperm migration	0.43	NS	Verberckmoes et al., 2002
					Progressive motility	0.66	$P < 0.01$	
					Membrane integrity	0.05	NS	
HF	10	52.8 - 69.9	163 - 268	Linear regression	Sperm-oviduct binding	-	$P < 0.05$	De Pauw et al., 2002*
SRW	8	55 - 83	171 - 441	Pearson's correlation	Hemi-zona pellucida binding	0.46	$P < 0001$	Fazeli et al., 1997
SRW	22	46.2 - 77.4	171 to 840	Multiple linear regression Spearman's rank correlation	Zona pellucida binding	0.50	$P < 0.05$	Zhang et al., 1998*
					Post-thaw linear motility	0.45-0.59	$P < 0.01$	
					Concentration motile spermatozoa after swim-up	0.43-0.63	$P < 0.01$	
4 SRW, 1 NRC[3]	5	68.6 - 76.6	1514 - 14583	Chi-square test for trend	Blastocyst development	-	$P < 0.001$	Shamsuddin and Larsson, 1993
HF	9	62.8 - 76.2 [††]	2181 - 9316	Linear regression	Cleavage rate	0.38	NS	Schneider et al., 1999
					Blastocyst development	-0.17	NS	
SRW	21	46.2 - 74.8	171 - 652	Spearman's rank correlation	Cleavage rate	0.59	$P = 0.0001$	Zhang et al., 1997
					Blastocyst development	0.35	$P = 0.028$	

[1]SRW = Swedish Red and White dairy bulls, [2]HF = Holstein friesian , [3]NRC = Norwegian Red Cattle.
The non-return rate (NRR) was determined at 56-59 d except for [†]at 90 d and [††]at 60-90 d.